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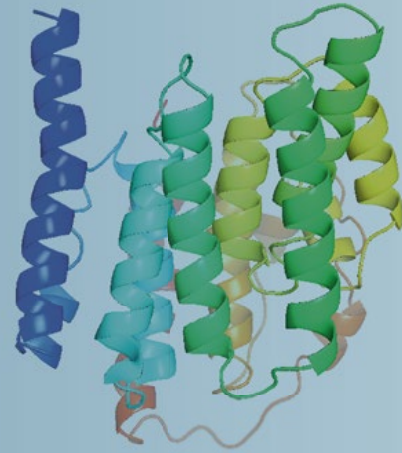
Sunil Thomas   *Editor*

Vaccine Design

Methods and Protocols, Volume 2. Vaccines for Veterinary Diseases

Second Edition

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Vaccine Design

Methods and Protocols, Volume 2. Vaccines for Veterinary Diseases

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Edited by

Sunil Thomas

Lankenau Institute for Medical Research, Wynnewood, PA, USA

Editor
Sunil Thomas
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Dedication

The healthcare and frontline workers who worked tirelessly taking care of COVID-19 patients.

Researchers who studied diligently the biology of SARS-CoV-2 and developed vaccines to protect against COVID-19.

Preface

Vaccinations have greatly reduced the burden of infectious diseases. Aggressive vaccination strategies have helped eradicate smallpox in humans and rinderpest, a serious disease of cattle. Vaccination has greatly reduced many pediatric infectious diseases. Vaccines not only protect the immunized but can also reduce disease among unimmunized individuals in the community through "herd protection." Vaccines have also led to increased production of fish and farm animals, thereby improving food security.

The development of vaccines has improved our understanding of immunology and the principles of immunity. This has led to the research and development of vaccines for cancer and neurodegenerative diseases.

The world's health and economy deteriorated since the first report of COVID-19 in China in December 2019. The pandemic has resulted in a huge interest in the development of vaccines. Even the skeptics were clamoring for early development of vaccines. Generally, vaccines take around 10–15 years to reach the clinic. Advances in the knowledge of molecular biology, immunology, and bioinformatics have led to the development of mRNA and adenovirus vector vaccines that are more efficacious than conventional vaccines. Collaboration at multiple levels led to the development and quick employment of COVID-19 vaccines in the clinic within a year of the observation of the disease, making it the quickest vaccines ever to be developed and deployed.

In 2016, we published the first edition of the book *Vaccine Design: Methods and Protocols.* Volume 1: *Vaccines for Human Diseases* and Volume 2: *Vaccines for Veterinary Diseases.* The books were a tremendous success.

The *Methods in Molecular Biology* series *Vaccine Design: Methods and Protocols*, Second Edition, contains 87 chapters in three volumes. *Volume 1: Vaccines for Human Diseases* has an introductory section on future challenges for vaccinologists, the immunological mechanism of vaccines and the principles of vaccine design. The design of human vaccines for viral, bacterial, fungal, and parasitic diseases as well as vaccines for tumors is also described in this volume. Volume 2: *Vaccines for Veterinary Diseases* includes vaccines for farm animals and fishes. Volume 3: *Resources for Vaccine Development* includes chapters on vaccine adjuvants, vaccine vectors and production, vaccine delivery systems, vaccine bioinformatics, vaccine regulation, and intellectual property.

It has been 225 years since Edward Jenner vaccinated his first patient in 1796 to protect against smallpox. This book is a tribute to the pioneering effort of his work. The job of publishing the second edition of the book *Vaccine Design: Methods and Protocols* was assigned at a tough time. Most of the universities were closed due to COVID-19 immediately after I took up the assignment. Several of the authors, their collaborators, and families were infected with the virus while contributing to the book. Nevertheless, the authors completed their chapters within the stipulated time. I am extremely grateful to the authors for completing the task in spite of the hardship faced while contributing to the books. My sincere thanks to all the authors for contributing to *Vaccine Design: Methods and Protocols* (Edition 2); Volume 1: *Vaccines for Human Diseases*; Volume 2: *Vaccines for Veterinary Diseases*; and Volume 3: *Resources for Vaccine Development.* I would also like to thank the series editor of *Methods in Molecular Biology*™, Prof. John M. Walker, for giving me the opportunity to edit this book. My profound thanks to my parents Thomas and Thresy, wife

Jyothi for the encouragement and support, and also our twins Teresa and Thomas for patiently waiting for me while preparing the book. Working on the book was not an excuse for staying away from the laboratory. I made sure that my children were told about new exciting data generated in the laboratory and the advances in science published daily before bedtime.

Wynnewood, PA   *Sunil Thomas*

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Part I

Veterinary Vaccines: Introduction

Chapter 1

Challenges in Veterinary Vaccine Development

Sunil Thomas, Ann Abraham, Alina Rodríguez-Mallon, Sasimanas Unajak, and John P. Bannantine

Abstract

Animals provide food and clothing in addition to other value-added products. Changes in diet and lifestyle have increased the consumption and the use of animal products. Infectious diseases in animals are a major threat to global animal health and its welfare; their effective control is crucial for agronomic health, for safeguarding food security and also alleviating rural poverty. Development of vaccines has led to increased production of healthy poultry, livestock, and fish. Animal production increases have alleviated food insecurity. In addition, development of effective vaccines has led to healthier companion animals. However, challenges remain including climate change that has led to enhancement in vectors and pathogens that may lead to emergent diseases in animals. Preventing transmission of emerging infectious diseases at the animal–human interface is critically important for protecting the world population from epizootics and pandemics. Hence, there is a need to develop new vaccines to prevent diseases in animals. This review describes the broad challenges to be considered in the development of vaccines for animals.

Key words Veterinary vaccines, Fish vaccines, Challenges, Vaccination, Livestock, Companion animals, Poultry, Ticks, Bacteria, Virus, Parasites, Ectoparasites

1 Introduction

Veterinary vaccines are developed to increase production of livestock including cattle and poultry, improve the health of companion animals, and prevent animal-to-human transmission from domestic and wild animals. Aggressive vaccination strategies globally have eliminated rinderpest, a devastating cattle disease in 2011. This was the first livestock disease, to have ever been eradicated through vaccination efforts, and the global benefits of rinderpest eradication are estimated to be in the billions of dollars [1].

Vaccination strategies are required to eliminate diseases of livestock so as to increase the food security in a rapidly changing world due to climate changes. Increase in temperature will lead to proliferation of pathogens where some species may become more susceptible to a novel pathogen during heat stress [2].

2   Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-COV-2)

SARS-CoV-2 is responsible for the disease COVID-19 that has decimated the health and economy of our planet. The virus causes the disease not only in people but also in companion and wild animals. As yet we do not know why the virus has been highly successful in causing the pandemic within 3 months of its first report [3]. The virus primarily infects upper respiratory tracts in humans and gastroenteritis and respiratory infections in birds and mammals. Transmission occurs primarily through respiratory droplets from coughs and sneezes within a range of 6 feet.

Some of the early case-patients had a history of visiting the Huanan Seafood Wholesale Market, where wildlife mammals are sold, suggesting a zoonotic origin. The causative agent was rapidly isolated from patients and identified to be a coronavirus, now designated as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Its genome is closest to that of SARS–related coronaviruses from horseshoe bats, and its receptor-binding domain is closest to that of pangolin SARS viruses [4].

Since the surge of the COVID-19 disease caused by the coronavirus SARS-CoV-2 in humans, the disease has been reported in companion, farm, and wild animals. Reports suggest that companion animals including cats and dogs may have acquired COVID-19 from their owners and that the virus jumped between humans and minks on farms in Europe. Minks were infected following exposure from infected humans. Minks can act as a reservoir of SARS-CoV-2, passing the virus between them, and pose a risk for virus spill-over from mink to humans. People can then transmit this virus between them. Additionally, spill-back (human to mink transmission) can occur. It remains a concern when any animal virus spills into the human population, or when an animal population could contribute to amplifying and spreading a virus affecting humans. Millions of minks were culled in farms in Denmark and the Netherlands to prevent the spread of COVID-19 to humans [5, 6]. SARS-CoV-2 replicates efficiently in cats, tiger, lion, minks, ferrets, and golden hamsters [7–10]. As viruses move between human and animal populations, genetic modifications in the virus can occur. These changes can be identified through whole genome sequencing [11]. As yet there are no therapies and vaccines for COVID-19 for animals. As more documented cases of animal-to-human transmission occur, a need to develop vaccines to protect against SARS-CoV-2 in companion, farm and wild animals will follow. Several companies including Pfizer, Moderna and AstraZeneca have developed COVID-19 vaccines for humans. A cheaper variant could be developed for animal immunization.

3   Challenges in Development of Vaccines for Poultry

With rapid population expansion, urban development, and improved quality of life in most parts of the world, agricultural development is playing an increasingly important role in the global economy. Agricultural development is essential for food security and for bringing millions of people out of poverty and starvation to build and maintain a stable society. In Asia, the livestock industry is the most crucial part of agricultural development and has been growing at an unprecedented pace in the last few decades [12]. Poultry provides the cheapest source of animal protein. The rearing of poultry has increased over the years due to development of vaccines that protect against several diseases. However, there are poultry diseases that require vaccines so as to increase its production.

Avian Pathogenic *Escherichia coli* (APEC) is a pathogen of the chicken and other avian species. It is considered to be a member of the extra-intestinal pathogenic *E. coli* (ExPEC) along with human Uropathogenic (UPEC) and neonatal meningitis-associated *E. coli* (NMEC) that cause disease outside the intestine. APEC infection may occur in broiler (meat) chicken, turkey, and other egg-laying poultry. In the broiler chicken, APEC infections are considered to typically lead to colibacillosis; a syndrome that includes respiratory tract infection, air sacculitis, pericarditis, perihepatitis, splenomegaly, and swollen head syndrome. In mature laying hens, reproductive tract infection leading to salpingitis or salpingo-peritonitis syndrome (SPS) is common [13]. APEC threatens food security and impacts the poultry industry's economy. Economic losses may be due to decreased hatching rates, decreased egg production, lowered production, carcass condemnation at slaughter, and prophylaxis [14]. APEC is known to infect all types of birds at all different ages in poultry. The disease can be transmitted through the respiratory system and the gastrointestinal tract.

Different strains of APEC cause variations in severity of the disease. Some strains are deadly while others are harmless. In the acute septicemic form, mortality starts immediately and progress rapidly. Morbidity is not clearly seen and healthy birds may even die. A sign of infection is ruffled feathers, indications of fever, and additional symptoms, such as labored breathing, coughing, and diarrhea. It is necessary to be diagnosed by laboratory tests since coliform infections may be confused with other diseases. Currently, management and sanitation practices are the most ideal for decreasing the amounts of these types of organisms in the birds' environment. Birds are suited to defend against harmful infections when stress factors and other disease agents are reduced. Good ventilation, good litter, a clean environment, high quality feed and water will aid in fighting the disease for birds [15]. Various medications

induce different responses; therefore, it can be difficult to treat the condition. Determining the sensitivity to the various drugs will aid in administering the most beneficial drugs.

Most APEC strains possess some common virulence factors suggests that an effective vaccine against APEC is a viable option. The most important virulence factors that have been investigated over the years include type I and P fimbriae, aerobactin iron-acquisition system, and serum resistance traits. Despite the potential for developing an efficacious vaccine to combat this economically important poultry disease, several obstacles hinder such efforts. Those obstacles include the cost, vaccine delivery method and timing of vaccination as the birds should be immune to APEC by 21 days of age [16].

Mycoplasma gallisepticum causes severe inflammation and primarily infects trachea, lungs and air sacs in chickens. *M. gallisepticum* is an extracellular pathogen with a total lack of bacterial cell wall and have the ability to adhere and colonize in mucosal surface epithelium, resulting in inflammatory signs like coughing, tracheal rales, and sneezing. *Mycoplasma gallisepticum* infection is commonly designated as chronic respiratory disease (CRD) of chickens or air sac disease and infectious sinusitis of turkeys. The disease causes high mortality in young birds, stunted growth, and reduced feed efficiency. Many fowl become unfit for human consumption. Infectious sinusitis in turkeys causes swelling under the eyes and inflammation of respiratory organs. It negatively impacts growth and feed conversion. *Mycoplasma gallisepticum* affects many types of birds and is spread primarily through the egg. Hens become infected with microorganisms and the chick is infected when it hatches. The disease may also be transmitted via direct contact. Diagnosis of the disease is based on flock history, symptoms, and lesions. Blood tests can be used to determine whether a flock is infected. Eradicating the disease in chickens and turkeys is the only solution to decrease mycoplasmosis infections. Antibiotics have been used to treat the disease, with varying degrees of success [17].

The pathogenic mycoplasma cause worldwide economic losses to chicken farming due to downgrading of carcasses, decreased feed conversion efficiency, and reduced hatchability and egg production. The pathogen induces a profound immune dysregulation and setting the stage for disease manifestations in chickens' tracheal mucosa [18].

Live vaccines can provide significant protection from the pathogenic effects of *M. gallisepticum* infection. However, differing management practices, including vaccination procedures, can lead to significant variations in the efficacy of the same vaccine. The site of vaccine deposition has been shown to be one important factor significantly influencing the vaccination outcome. Vaccine applied to the eyes or sprayed on the head is significantly more effective

than when sprayed on the body. Vaccine application to the eyes is significantly more effective than nasal vaccination, and vaccine delivered through the oral cavity has a negligible contribution to overall vaccination outcome [19].

The Gram-negative rod bacteria *Pasteurella multocida* causes fowl cholera, which is a severe disease of poultry. It is seen either as acute or chronic forms, and the clinical signs vary depending on the form of the disease. Symptoms include depression, ruffled feathers, fever, anorexia, mucous discharge from the mouth, diarrhea, and an increased respiratory rate. Carrier birds play a major role in the transmission of fowl cholera [20].

Fowl cholera occurs in wide range of hosts including chickens, turkeys, pheasants, waterfowl, sparrows, and other free-flying birds. Due to the microorganism's persistence to survive in droppings, decaying carcasses, and soil, the pathogen can easily transmit to other birds through entry into the tissues in the mouth and the upper respiratory tract. The major sources of infection include excretions of diseased birds that contaminate water, soil, feed, etc., decaying carcasses, contaminated water supplies, and contaminated shoes or equipment. Diagnosis of the disease can be determined based upon flock history, symptoms, and postmortem lesions. Currently, there are no effective vaccines developed to treat the disease; administering antibiotics could lower the mortality. Good sanitation practices, including rodent control, proper disposal of dead birds, providing clean water, clean houses and equipment, confinement of birds away from wild birds and animals, an vacancy of contaminated ranges or yards for at least 3 months, are instrumental in preventing the disease. Drugs can be administered to decrease the spread, however, affected birds remain carriers and the disease continues to recur when treatment is discontinued [21].

Commercial vaccines against fowl cholera currently include attenuated live vaccines and inactivated vaccines. The protective efficacy of these vaccines, however, is not ideal. Attenuated live vaccines have considerable side effects, can cause excretion virion, and are difficult to store. Inactivated vaccines can cause poor immunogenicity and short-term immunoprotection. Nevertheless, novel vaccines such as genetically engineered subunit vaccines and DNA vaccines have represented a promising approach toward the prevention of hemorrhagic septicemia caused by *P. multocida*. Recombinant subunit vaccine, has many advantages including better safety and low manufacturing costs, and has become one of hotspots in the field of vaccine research [22].

Necrotic enteritis is an acute disease which destroys the intestinal lining of the digestive tract. The organism responsible for the disease is *Clostridium perfringens* that produces toxins harmful to the intestinal lining. Transmission is predicted to be by oral contact with the droppings of infected birds. The disease is very deadly,

even for healthy birds. It is known that healthy birds may become acutely depressed and die within hours of infection. The lower half of the small intestine is mainly infected but the entire length of the tract can be involved. It is dilated, filled with dark offensive fluid, and has a cauliflower-like membrane that involves the mucosa. Despite the effective treatments in place, such as bacitracin, virginiamycin, preventative medication, and vitamin treatment, a vaccine has not been developed to mitigate the spread of necrotic enteritis. Moribund birds are removed promptly, because they can serve as a source of toxicosis or infection due to cannibalism [23].

There are currently no necrotic enteritis vaccines commercially available for use in broiler birds, the most important target population. Immunizing ability for protection against necrotic enteritis was associated with infection with virulent rather than with avirulent *C. perfringens* strains [24]. There is evidence that immunization with single proteins is not protective against severe challenge and that combinations of different antigens are needed. Most published studies have used multiple dosage vaccination regimens that are not relevant for practical use in the broiler industry. Single vaccination regimens for one-day-old chicks appear to be non-protective [25].

Ulcerative enteritis (quail disease) is an acute or chronic infection caused by *Clostridium colinum*, which infects game birds, chickens, turkeys, quail, and other domestic fowl. The infection is transmitted by the droppings from sick or carrier birds to healthy birds. The microorganism can resist disinfectants and varying environmental conditions. Acute infections will lead to immediate death in birds; whereas, chronic infections affect birds severely. The entire intestinal tract is lined with ulcers, with the lower portion being the most affected. The ulcers will often perforate, leading to peritonitis. Bacitracin and penicillin are the most effective drugs in treatment and prevention of the disease [26].

Other diseases of notable importance that cause diseases in poultry includes: pullorum disease in chickens and turkey caused by *Salmonella pullorum* [27]; fowl typhoid caused by *Salmonella enterica* Gallinarum [28]; botulism caused by *Clostridium botulinum* [29]; Omphalitis caused by a mixture of bacterial infections (coliforms, *Staphylococci*, *Pseudomonas* spp., and *Proteus* spp) [30]; Erysipelas caused by the bacteria *Erysipelothrix rhusiopathiae* [31]. None of these diseases have vaccines to provide protection to poultry.

4   Challenges in the Development oF Vaccines for Companion Animals

The companion animals include dogs and cats that are considered being part of the family. They provide emotional support as well as security to the family. In addition, guide dogs help people with

disabilities. Due to the large prevalence of dogs and cats in households, shelters, and the streets, it is essential to understand and prevent infectious diseases from affecting the companion animals, as well as reducing the risk of transmitting diseases to their owners.

Heartworm disease (dirofilarosis), caused by the filarial parasite *Dirofilaria immitis*, primarily affects the pulmonary arteries, producing inflammation, vascular dysfunction, and pulmonary hypertension. It is a major disease in dogs. Mosquitoes can serve as intermediate hosts; Aedes, Anopheles, and Culex are the most common genera acting as vectors. Wild animal reservoirs include wolves, coyotes, foxes, California gray seals, sea lions, and raccoons. The presence of *D. immitis* in dogs constitutes a risk for the human population. The human host is the causative agent of the pulmonary dirofilariasis and in many cases produces benign pulmonary nodules which can initially be misidentified as malignant tumors [32]. A vaccine would provide ease and accessibility for veterinarians to treat heartworm disease in dogs.

Toxoplasmosis is caused by the single cell protozoan parasite, *Toxoplasma gondii* and is primarily transmitted by oocysts in the feces of domesticated and wild cats. Other hosts contract the disease by ingesting infective oocysts from cat feces or contaminated soil, water, or other materials. The oocysts that are shed by cats are highly infective for most nonfeline mammalian hosts. Toxoplasmosis is mainly prevented by avoiding exposure to cat feces and handling and preparing food with care and caution. Dogs can also serve as intermediary hosts of *T. gondii* [33]. More than 40 million people in the United States may be infected with the Toxoplasma parasite. The Toxoplasma parasite can persist for long periods of time in the bodies of humans (and other animals), possibly even for a lifetime. Of those who are infected however, very few have symptoms because a healthy person's immune system usually keeps the parasite from causing illness (CDC.gov). Human infection can be avoided by drinking and eating pasteurized dairy products and meat which is properly cooked [33].

A very important animal in the life cycle of *T. gondii* and the epidemiology of the disease is the cat. Following a primary infection, cats will shed millions of oocysts in their feces that can survive for 12–18 months in the environment, depending on climactic conditions, and are an important source of infection for grazing animals. Therefore, a range of different veterinary vaccines are required to help control T. gondii infection which include vaccines to prevent congenital toxoplasmosis, reduce or eliminate tissue cysts and to prevent oocyst shedding in cats [34].

In a study, cats were vaccinated with the T-263 vaccine (Toxoplasma vaccine) and the efficacy of the vaccine was measured indirectly by examining seroprevalence of other intermediate animal hosts, including the farmed pigs within the study farms. The results showed a decrease in seroprevalence within the pig population

implying that vaccinating the cats had reduced the shedding of *T. gondii* oocysts into the environment and therefore reduced the source of infectious material for the intermediate animal hosts in this area [35]. Further analysis of this study showed that the decrease in T. gondii seroprevalence observed in the farm pigs was related to the number of cats on the farm, oocyst survival in the environment and the vaccination of cats with the T-263 vaccine [34].

Giardiasis is caused by the protozoan parasite *Giardia duodenalis* and infect canines, felines, and humans. *Giardia* cysts and trophozoites are the main modes of transmission when they are shed in the feces of infected humans or animals. The fecal-oral route is the primary avenue of transmission after consumption of contaminated water or food. Prevention of the disease comprises of proper sanitation of water sources, prompt removal of fecal material, avoidance of consuming contaminated water or feces, and the disinfection of kennels [36]. Efficient vaccines against Giardia are not available. Giardia undergoes antigenic variation; through this mechanism, parasites can avoid the host's immune defenses, causing chronic infections and/or re-infections. Antigenic variation is characterized by a continuous switch in the expression of members of a homologous family of genes encoding surface antigens. Giardia also infects humans; immunization of dogs with a highly efficient vaccine would decrease the percentage of infected children in the community [37].

5   Challenges in the Development of Vaccines for Pack Animals

5.1   Llamas and Alpacas

Mycoplasma haemolamae is a hemotropic mycoplasma that affects the red blood cells of llamas and alpacas. Animals infected with *M. haemolamae* exhibit symptoms of mild to severe anemia, lethargy, depression, and fever [38]. Anemia is reported in infected animals that are immunosuppressed, stressed, debilitated, or suffering from a concurrent illness [39]. Death is even possible in heavily infected llamas and alpacas. Transmission of the bacterium is unknown but it is hypothesized to be spread by insect vectors [38].

Bovine viral diarrhea virus (BVDV) is a diverse group of viruses that affect multiple organ systems, suppresses the mammalian hosts' immune system, and transmits by direct and indirect routes. It is an enveloped, single-stranded RNA virus and is a member of the genus *Pestivirus* in the family Flavivirdae. Since BVDV is a RNA virus, genetic mutations occur frequently; therefore, genetic, antigenic, and pathogenic variations are prevalent. Infections of BVDV have been identified in Old and New World camelids. Twenty percent have been reported in both North and South America

and herd-level prevalence is 25% where 63 alpaca herds were tested in the U.S. When infected with BVDV, camelids show very few or no clinical signs of disease [40].

Other infectious diseases affecting llamas and alpacas are equine herpesvirus, Eastern equine encephalomyelitis, bluetongue virus, and alpaca respiratory coronavirus. Vaccinations have not been developed for stated diseases and it is imperative for veterinarians and vaccinologists to find solutions to alleviate these animals of suffering from infectious diseases.

5.2 Yak

Yak are indigenous to the regions surrounding the Himalayas including Tibet, Nepal, Bhutan, Indian states of Himachal Pradesh, Sikkim, Arunachal Pradesh and Ladakh, Central Asia and Mongolia. Many diseases infecting yaks are linked with stress from the feed deficit in winter and early spring and from weather conditions. Bacterial diseases such as calf scours, contagious bovine pleuropneumonia, chlamydia infection, leptospirosis, lymphadenitis, mastitis, pasteurellosis, salmonellosis, tetanus, tuberculosis, blackquarter, *Coxiella burnetti*, kerotoconjunctivitis, and camphylobacterosis are common among yaks. Viral diseases such as foot and mouth disease, infectious bovine rhinotracheitis, viral diarrhea/mucosal disease, vesicular stomatitis, calf diphtheria, and parainfluenza, bovine Herpes virus, are also reported in yak. However, vaccinations are rarely used by the herdsman to treat the infections. Due to the remoteness and inaccessibility of areas flocked with yaks, conventional health services are limited [41]. Vaccinations to the diseases of yak could improve the livelihood of people in the region.

5.3 Buffalo

Buffalos are used in farming, milk production, transportation, and a major source of income for people in rural Asia. Buffaloes are easy to maintain than other cattle. Buffalo meat is lower in fat compared to cattle meat. India has the largest buffalo population, with many rural households owning a buffalo. Since India is a large buffalo meat exporter, the health and safety of the buffaloes are very critical for the economy [42].

Infectious diseases including leptospirosis, bovine tuberculosis, rotavirus, bovine viral diarrhea virus, and fasciolosis are major diseases impacting buffalo; however, published water buffalo-specific research is limited since the animal is not important in many countries and the epidemiology of these diseases is not fully understood in domesticated water buffalo.

Bovine leptospirosis is caused by the pathogen *Leptospira* and leads to chronic infections. Pyrexia, hemolytic anemia, hemoglobinuria, jaundice, meningitis, and even death are symptoms of leptospirosis. Infection occurs in the mucous membranes of the eye, mouth, nose, or genital tract. Infection in pregnant females can

lead to abortion and other neonatal diseases. Damage to the endothelial cells of small blood vessels is known to be the primary lesion due to leptospirosis.

Bovine tuberculosis (BTb) is caused by *Mycobacterium bovis* and is closely related to M. tuberculosis in humans. *M. bovis* is primarily transmitted from animal to animal by inhalation or ingestion and skin inoculation. Lesions due to BTb in buffalo are tuberculosis mass in the lungs and lymph nodes. The development of a vaccine will relieve economic and public health burdens in developing countries.

Rotavirus attacks the villi of the small intestine, which suppresses the absorption of nutrients into the animal's body leading to dehydration. Symptoms include runny diarrhea, dehydration, and loss of appetite. Transmission occurs upon contact with infected feces. The virus is persistent and can survive for several months and resist several disinfectants. There is no specific treatment for the infection but replacing lost fluids and restoring the body's balance with important electrolytes is key to managing the virus. Antibiotics are viable in the presence of a secondary infection due to bacteria, but it is not recommended. Vaccines can be used for prevention; however, the vaccine only provides protection for 3–4 days in newly born calves.

Bovine viral diarrhea virus (BVDV) infects buffaloes and causes respiratory and reproductive illnesses. Clinical signs of the virus include diarrhea, mucosal disease, and reproduction dysfunctions such as abortion, teratogenesis, embryo resorption, fetal mummification, and stillbirth. Modified live virus (MLV) vaccines and killed virus (KV) vaccines are available to use against infection; however, MLV vaccines are not stable in varying temperatures and can be easily deactivated by some chemicals. KV vaccines, alternately, are stable in varying temperatures and are not easily deactivated by chemicals, but are more expensive.

Fasciolosis is caused by a trematode *Fasciola* species and is known to occur in tropical and subtropical areas in Africa, and Asia. Buffaloes are susceptible to infection, especially in animals more than 3 months old since they eat or graze on farm grasses, which exposes them to a greater chance of infection. Animals become infected when raw fresh-water vegetation in ingested. Fasciolosis can be categorized as subacute and chronic fasciolosis. Subacute cases survive 7–10 weeks with great liver damage and the animal eventually dies from hemorrhage and anemia. Chronic fasciolosis includes symptoms of anemia, wasting, submandibular edema, and decreased milk production. As yet there are no vaccines for the above diseases. Effective vaccinations would increase the production and economy of the regions relying on Buffalo for its economic activities [43].

6   Challenges in the Development of Vaccines for Cattle

In this section, we will examine vaccine challenges for ruminants with an emphasis on cattle diseases (excluding buffalo). There are numerous considerations and challenges related to vaccination of cattle and other ruminants. There are several bacterial and viral diseases that dairy and beef cattle are currently vaccinated against (Table 1). Aside from the obvious issues of funding needed for testing vaccines in large animals such as cattle and sheep, along with the difficulty to induce a rapid and long-lasting protective immunity after a single dose of vaccine for nearly all cattle diseases, we focus on other logistical challenges. These include vaccine delivery considerations and the annulling effects of vaccines from outside agents as important issues as well as the timing of vaccine administration.

Table 1
Diseases of cattle that are commonly vaccinated against

Disease	Agent
Anthrax	*Bacillus anthracis*
Blackleg	*Clostridium chauvoei*
Black's disease	*Clostridium novyi*
Bovine viral diarrhea	Bovine viral diarrhea virus
Brucellosis	*Brucella abortus*
Enterotoxemia	*Clostridium perfingens*
Infectious bovine rhinotracheitis (IBR)	Bovine herpes virus 1
Leptospirosis	*Leptospira hardjo bovis*
Malignant edema	*Clostridium septicum*
Mastitis	*Staphylococcus aureus, Escherichia coli*
Pinkeye (keratoconjunctivitis)	*Moraxella bovis*
Pneumonia	*Mannheimia haemolytica*
Redwater	*Clostridium haemolyticum*
Salmonellosis	*Salmonella typhimurium*
Shipping fever	*Pasteurella* species
Vibriosis	*Campylobacter fetus*
Trichomoniasis	Protozoal etiology
Bovine respiratory disease complex	*Pasteurella multocida, Mannheimia haemolytica, Histophilus somni*

**6.1 Timing of
Vaccination**

Considerations on the best time to vaccinate can be divided into age related issues or circumstance related issues. For example, the life stages of dairy and beef cattle include neonatal, yearling, 2-year old, and adult. To be most effective and account for stages when the animal may be most susceptible to infection, some vaccines should be administered to the neonatal calf whereas others can be administered to the adult cow. Vaccines for Johne's disease are usually administered to neonatal calves up to 1 month of age [44] while heifer calves at 4–12 months are vaccinated against Brucellosis [45]. For Johne's disease, the newborn calf is most susceptible to infection while the adult cows are relatively resistant to the disease [46]. Viral vaccines containing either bovine rotavirus or bovine coronavirus can be given orally to newborn calves [47, 48]. However, the oral modified live virus (MLV) vaccine should be given prior to ingestion of colostrum or it will not be activated [48]. Passive immunity using injectable rotavirus/coronavirus/*E. coli* combinatorial vaccine in the dam prior to calving is the method of choice for protecting calves from these diseases by stimulating colostral antibody production.

Adult cows, 3 years and older, are generally vaccinated for viral diseases. These include infectious bovine rhinotracheitis (IBR), parainfluenza-3 (PI-3), bovine virus diarrhea (BVD), and bovine respiratory syncytial virus (BRSV). These vaccinations are often performed during the pre-weaning period, but occasionally as soon as 3–6 months of age. Animal health professionals advise use of these vaccines in selected herds with a history of these diseases, but this should be implemented cautiously in young calves. The injectable IBR vaccine, if administered to calves less than 5 days of age, could result in an adverse BHV-1 infection not seen with intranasally delivered vaccines [49]. Another study also suggested that a protective immune response will develop if intranasal respiratory vaccines are administered to calves for BHV-1 [50]. A similar finding was obtained with neonatal calves vaccinated intranasally with the BRSV/IBR/PI-3 vaccine, showing that it may be more beneficial than standard injectable vaccines in calves [51]. However, intranasal vaccines generally have a shorter duration of immunity than injectable vaccines [51], a factor to be considered in any vaccination regimen.

Bacterial diseases including those caused by *Leptospira*, *Clostridia*, *Escherichia coli*, *Staphylococcus*-causing mastitis, and calf diarrheal diseases can be controlled by vaccination during the lactation period and/or the dry period. Calves are usually vaccinated for the major clostridial diseases prior to pasture turnout with a 7-way clostridial vaccine. Some clostridial vaccines can be given to newborn calves but immunity will be improved if vaccination is delayed until branding or pasture grazing age. However, MLV vaccines against viral pathogens, especially for IBR, should not be used in pregnant cows as the vaccines have been reported to cause

abortions [52]. A separate problem with the IBR vaccines is that the antibody response generated is serologically indistinguishable from the BHV-1 virus known to cause the disease, which confounds diagnostic testing [53]. With these complications, a veterinarian should be consulted before using any MLV in pregnant cows.

Vaccines can be unsuccessful at eliciting protective antibodies when administered to young calves. Although very young animals have a functional immune system that can respond to vaccines and antigens, they are immunologically immature compared to older animals and thus may not respond to the vaccine or antigen as effectively. Antibodies acquired passively from the dam through immunoglobin-rich colostrum protect the calf from many infectious diseases, but they may also inhibit the antigens comprising the vaccine. This transferred maternal antibody interference is a primary reason for not vaccinating very young calves against some infectious diseases [54, 55]. Nonetheless, there are vaccines that can produce an effective immune response, even in newborn animals [54, 56].

Another consideration is when to administer a vaccine based on circumstances related primarily to purchase and transport of animals or disease outbreaks. The shipping of weaned calves from pasture to feedlot without vaccinating is a questionable practice that can result in increased illness and death [57]. Beef cattle producers often want their newly purchased livestock vaccinated as soon as the hoofs touch their farm, but in reality, they are immune-stressed after transport and should be acclimated prior to vaccination [58]. Instead, studies suggest that weaning beef calves and then vaccinating them 40–60 days before arrival on the feedlot will reduce morbidity and mortality [59, 60]. As a potential alternative, once feeder cattle are acclimated, they can be vaccinated soon after arrival in the feedlot. There are very rare exceptions to these guidelines [61]. Some disease outbreaks occur at consistent times throughout the year or in repeatable multi-year cycles and this can also play a role in when to vaccinate. Recorded intervals between outbreaks may yield insight into the management stresses that are introduced cyclically and correcting these should have a positive effect on any vaccination program.

Animal producers also need to consider any negative effects of administering multiple vaccines to an animal. More specifically, there is the potential for two vaccines, when administered together, to annul their protective effects. For example, vaccination with a modified live BHV-1 vaccine blocked the protective response to a *Mannheimia haemolytica* vaccine [62]. This bacterium is a major cause of pneumonia in cattle. More recent studies examined the serologic responses to a *Mannheimia haemolytica* killed cell vaccine with co-administration of a MLV against BHV-1 which appeared to abrogate antibody responses; however, immunodominance was not

observed if the MLV was administered intranasally [63]. The cancelling effects of antigen responses from competing vaccines have been shown in a few other examples as well [64]. Additional research is needed to better understand these negative effects and how they can be mitigated in vaccine regimens for cattle.

Generally, a single modified live vaccine is recommended for feeder calves unless special circumstances exist. High risk calves could benefit from a vaccine delivered intranasally followed 2 weeks later with the traditional injectable vaccine. The oral MLV vaccine should be given 30 min prior to ingestion of colostrum or it will be inactivated. Some veterinarians prefer to use injectable rotavirus/coronavirus/*E. coli* in the dam prior to calving and depend on colostral antibodies to protect calves. It is best to minimize the number of vaccines given at one time as much as possible. Multiple vaccinations can cause neck soreness while multiple Gram-negative vaccines may cause a fever spike in cattle from excess lipopolysaccharide (endotoxin). This effect may manifest itself by observing the animal not eating for a brief period of time.

Most MLV vaccines should not be given to naive pregnant cattle because they can invade the fetus and cause birth defects or abortion [65]. Examples include the injectable modified live infectious bovine rhinotracheitis and bovine virus diarrhea vaccines. Furthermore, Brucellosis is a reproductive disease, causing abortions and infertility. It is recommended that heifers (female calves) between the ages of 4 and 12 months be vaccinated against *Brucella abortus*. In general, abortions can be caused by infection of the placenta, sudden death of the fetus, or by inflammation of the ovary. The goal of vaccination against reproductive diseases is to minimize the amount and duration of the viremia or septicemia, and it ideally should prevent the pathogen from crossing the placenta. Modified live vaccines generally produce a higher level of immunity than killed vaccines, but may have a degree of risk when given to either pregnant or highly stressed cattle. These combined factors make it difficult to achieve protection against reproductive diseases.

6.2 Vaccine Safety and Delivery Considerations

Commercial and field applications of cattle, sheep, goat, and camel vaccines are also limited due to formulation and delivery constraints.

Safety concerns, related to live or modified live vaccines, include the risk of lingering virulence, reversion to the pathogenic wild type agent and potential for unintended consequences if non-target species are exposed to the vaccines. The potential for zoonotic infection from needle sticks is a further concern. These scenarios have resulted in strict regulatory requirements for live-attenuated vaccine approval. Another issue with live-attenuated vaccines or killed whole cell extracts (i.e., Johne's disease and Foot and Mouth Disease) is that they can confound disease

surveillance when serological testing of cattle is performed since false positives could result in the loss of a country's disease-free status [66, 67]. For Johne's disease, the vaccine can only be administered by a veterinarian and must be done according to state regulations.

Whole cell inactivated vaccines are considered safer than modified live vaccines, but their inability to trigger Th1 immune responses through infection usually results in predominantly a humoral immune response without any cell-mediated immunity, potentially making them less effective. This consideration is especially important when dealing with intracellular pathogens, including *Mycobacterium*, *Brucella*, and viral agents [68, 69]. Consequently, inactivated or killed cells may require the use of immunostimulatory adjuvants with multiple dosing to achieve a sustained and desired level of protective immunity. Furthermore, oral delivery of inactivated microorganisms, or more likely subunit antigens, may require incorporation of specific carrier systems such as microparticles or nanoparticles [70]. The necessity for immunological adjuvants, carrier systems and multiple dosing highlights the need for specific formulation strategies for these antigens. Indeed, a few commercial vaccines, based on killed, modified live or inactivated microorganisms to protect against bovine respiratory diseases, scours and clostridial diseases, are in use for beef and dairy cattle.

In some cases, prophylactic treatment simultaneous with vaccination is desired during a disease outbreak. This situation arises particularly with anthrax outbreaks in cattle herds [71]. However, delivery of the commonly used anthrax live spore vaccine [72] in conjunction with antibiotic treatment of cattle during an outbreak is not recommended due to an inhibitory effect on the live vaccine [71, 73]. This limitation can be overcome if using a nonliving subunit vaccine composed of recombinant and immunogenic proteins from *Bacillus anthracis*, the disease causing agent [74]. The nonliving subunit vaccine is still under development, but if proven effective in the target host, it could circumvent other shortcomings inherent in the live spore vaccine including residual virulence resulting in death of some vaccinated animals [75], batch-to-batch variation resulting in inconsistent immune stimulation, and potential environmental contamination during production.

6.3 Economic Considerations

The major factors driving the growth of animal vaccines market include growth in livestock population and repeated breakouts of livestock diseases; increasing adoption of companion animals; rising incidence of zoonotic diseases; initiatives by various government agencies, animal associations, and leading players; and the introduction of new types of vaccines. Based on the type, the veterinary vaccines market is segmented into porcine vaccines, poultry vaccines, livestock vaccines, companion animal vaccines, aquaculture

vaccines, and other animal vaccines. The porcine vaccines segment is expected to account for the largest share of the animal vaccines market. The dominant share of this segment can be attributed to the increasing porcine population, growing awareness about animal vaccination, and outbreaks of porcine diseases such as PRRS (Porcine Reproductive & Respiratory Syndrome), swine influenza, and Auzeszkys Disease [76].

The global animal vaccines market size was worth at USD 8.2 billion in 2018. Increase in livestock populace, rising animal husbandry combined with commercialization of animal products are essential development drivers for this market. The global veterinary vaccines market is projected to reach USD 11.3 billion by 2025 [77].

Oral delivery of vaccines to animals, particularly to large farm animals such as cattle, sheep, and goats, is challenging to scale up of vaccine production; consolidated efforts by veterinary, and biotechnology researchers are required for successful development, packaging and implementation.

7   Challenges in Development of Vaccines for Sheep and Goat

There are very few vaccines to protect against bacterial, viral and parasitic diseases in sheep and goats. Sheep and goats are the major livestock of developing countries especially in Asia and Africa. Sheep and goats provide a vital source of food, income and security. The challenges include developing vaccine against multiple diseases, and they should be economical to the community [78].

Peste des petits ruminants (PPR) is a highly contagious and economically important viral disease affecting goats, sheep, and wild ruminants. The causal virus, a member of the Morbillivirus genus in the family Paramyxoviridae, preferentially replicates in lymphoid tissues and epithelial tissue of the GI and respiratory tracts, where it produces characteristic lesions. The disease is currently circulating in Asian and African countries, creating problems in small ruminant farming. Current control of the disease mainly includes isolation and disinfection of the contaminated environment, and administration of a live-attenuated vaccine, which provides a strong immunity. The current vaccination for Peste des Petits ruminants PPR is stalled by myriad challenges and continuous endemicity of pneumonia due to fulminant bacteria complication in goats. Mass vaccination of sheep and goats in endemic countries might be a pragmatic approach to control PPR in the first phase of disease eradication. Maintenance of cold chain for vaccine efficacy has proven difficult in subtropical countries. A thermostable live-attenuated conventional or recombinant vaccine is a way to avoid cold chain-associated problems in tropical and subtropical countries [78].

Parasitic diseases pose a threat to the health of sheep and goat, especially the gastrointestinal tract, reproductive performance, growth rates, meat, fiber, and milk. General clinical signs of infection include diarrhea, weight loss, loss of appetite, and reduced reproductive performance. Goats are more prone to internal parasitic infection compared to sheep. The most common internal parasite are different species of roundworms (*Teladorsagia circumcincta*, *Haemonchus contortus*, and *Trichostrongylus colubriformis*). One of the major roundworm that causes disease is *Haemonchus contortus*, which reproduces through egg-laying and causes anemia, edema (swelling of lower jaw), protein loss, lethargy and death. Vaccines are a promising control strategy against parasites; however, the extensive genetic variation and immunoregulatory characteristics of parasites obviously hinder vaccine development. Therefore, for the discovery of an effective, safe, and durable vaccine against *H. contortus*, researchers have been focusing on the development of molecular-based vaccine targets that are efficient against *H. contortus* infection and the utilization of advanced molecular approaches for structural and functional studies on vaccine candidates [79].

Coccidiosis is caused by microscopic protozoan parasites called coccidia. Coccidia are host-specific and each animal species is susceptible to infection with various coccidia species. In sheep and goats, coccidiosis is caused by the genus *Eimeria* [80]. Coccidia damages the lining of the small intestine and causes weight loss, stunted growth, diarrhea, dehydration, fever, anemia, and wool breakage. Coccidia occurs in animals in confinement or intensive grazing systems due to poor sanitation, overcrowding, and stress. Prevention protocols require establishing good sanitation techniques, providing clean water, rotating pastures, and avoiding overstocked pens [81]. Live-attenuated *E. ninakohlyakimovae* oocysts orally administered showed almost no pathogenicity but enough immunogenicity in terms of immunoprotection. However, vaccinated animals still shed low amounts of oocysts, guaranteeing environmental contamination and consecutive booster infections to sustain ongoing immunity [82].

Listeriosis is a bacterial infection which is spread by bacteria in the soil and GI tracts of mammals. It is caused by the bacteria *Listeria monocytogenes*. Sheep and goats are prone to infection when grazing pastures contaminated by feces infected by the bacteria or by entering tissue via wound or inhalation. Abortive diseases, such as toxoplasmosis, leptospirosis, and Q fever, are significant diseases affecting female sheep or goats, as well as their offspring. Abortive diseases, due to infectious or noninfectious agents, result in the death or deformation of babies. Leptospirosis causes abortion in goats (sheep are less susceptible). Transmission occurs in standing water that is infected by bacteria (*Leptospira interrogans*). Q fever is caused by the bacteria *Coxiella burnetti*

and cause abortion in sheep and goats. The bacteria is spread via the milk, urine, feces, placental tissue, amniotic fluid, and the air. Symptoms include anorexia, abortion, and lesions. Control of the disease includes oral tetracycline, separating pregnant animals from the rest of the herd, and burning/burying reproductive waste. Humans (veterinarians, farmers, researchers) can contract the disease when aiding the birthing process [81]. Coxevac is a vaccine that contains inactivated *Coxiella burnetti* bacteria. Vaccine-derived *C. burnetii* DNA may be excreted in milk after vaccination. Within hours and up to 9 days after vaccination with Coxevac, vaccine-derived *C. burnetii* DNA can be detected in the milk of dairy goats. A 2-week interval was introduced between vaccination and bulk tank milk testing to identify infected farms [83].

Mastitis is a bacterial infection that causes inflammation of the mammary glands, especially udder damage. Diagnosis of mastitis is determined by the physical examination of the udder or a sample of milk from the infected gland. Mammary glands become warm, swollen, and painful and lead to abnormal milk production. Treatment of mastitis includes intramammary and systemic antibiotics. Other diseases impacting sheep and goats are polioencephalomacia, white muscle disease, pregnancy toxemia, and lactic acidosis, which vaccines have not been developed for the eradication of the disease. Despite the preventative measures and control protocol implemented at farms and herd houses, vaccines will be crucial in alleviating the stress on animals, as well veterinarians and farmers, in treating these infectious diseases in sheep and goats [81].

8   Challenges in the Development of Vaccines Against Ectoparasites

A parasite is an organism that lives on or within a host organism and feeds at its expense. There are three major classes of parasites: protozoa, helminths, and ectoparasites. The term "ectoparasites" includes broadly parasites that depend on the blood of a host for food and survival. Most invertebrate ectoparasites are arthropods; insects and arachnids typically parasitizing terrestrial animals, while crustaceans are fish ectoparasites. In this sense, mosquitoes are ectoparasites, however, this term is usually employed with a more restricted meaning referring to organisms such as ticks, fleas, lice, and mites, which attach to or burrow into the host skin and remain there for relatively long time periods. In this chapter, ectoparasites will be referred with the broader meaning of the term.

Ectoparasites are themselves important causes of disease because they can produce anemia, detrimental immune reactions such as hypersensitivity and anaphylaxis, irritability, dermatitis, skin necrosis, low weight gains or weight loss which are particularly important in livestock, secondary infections, local hemorrhages, inoculation of toxins and occasionally exsanguination

[84]. Nevertheless, they are even more important as vectors, or transmitters, of many different pathogens that produce enormous morbidity and mortality from the diseases they cause [85].

Ectoparasite control has been fundamentally based on the use of chemicals which includes organochlorines, organophosphates, amidines, and pyrethroids among others [86]. However, each country applies different prevention/treatment programs. In many of them, especially in poor countries, farmers usually do not have official programs to control ectoparasites. They can use indiscriminately all kinds of chemical products with little governmental control. In this situation, both intensive use and under dosage lead to the establishment of drug resistant ectoparasite strains. In the last case, the incorrect drug administration is a practice that exposes ectoparasites to sub-therapeutic levels increasing the possibility of selecting for chemical resistant heterozygotes [87]. These bad practices coupled with the lack of systematic resistance monitoring system imply in many cases, the ignorance of efficacy performance of chemicals in field conditions. For example, resistant and multi-resistant tick strains have been reported in many countries as a consequence of the intensive or incorrect use of acaricides [88–92]. This increased tick resistance to available chemicals worldwide compared to the limited capacity to develop new substances, worryingly, could lead our supply of effective compounds will be exhausted if other management alternatives are not applied [86]. Therefore, it is important to involve a local veterinarian services in all ectoparasite control programs in order to guarantee the rational employment of chemicals.

Vaccination is still the most desirable means of combating infectious agents based mainly in world experience with anti-microbial vaccines [93]. Humans, pets and livestock are routinely vaccinated against many viral and bacterial pathogens; most of these vaccines are available commercially as bacterins, toxoids, and killed or attenuated viruses. The use of these vaccines has significantly increased the human life expectancy and enhanced livestock productivity by reducing, or in some cases eliminating, morbidity and mortality due to specific etiological agents. In contrast to the established immunological controls for viral and bacterial pathogens, there is no vaccine available for any human parasite, and there are few reliable available vaccines on a large scale for the immuno-prophylaxis of parasitic diseases of livestock [94, 95] despite parasitic infestations are the cause for billions of dollars in annual losses and medication costs in the livestock.

The reason for absence of vaccines for parasites is due to the biochemical complexity of parasites and the multiple stages in their life cycles which make difficult the identification of protective antigens. In dealing with complex multi-cellular organisms, as ectoparasites, it is not obvious to which antigens should be directed effective immune responses. It is also important to understand

that ectoparasites develop their life cycles outside of the parasitized organism and contact with the host immune system is only during feeding. In addition, other challenges include, the high number of different ectoparasite species such as ticks (more than 900 described species), their capacity to parasitize every class of terrestrial vertebrates and their capacity of feeding intermittently and using different hosts [96] are characteristics which complicate the challenge for achieving ectoparasite control through immunizations.

Traditionally, the attention of anti-ectoparasite vaccine developers had focused on molecules exposed on parasite surface or secreted by the parasite, assuming that accessibility of the host immune system to the target molecule(s) was an absolute requirement. Many attempts using this kind of antigens against ticks did not provide sufficient protection [97–99]. Low immunogenicity or no protections despite high antigenicity were the results of these studies. After millions of years, parasites and hosts have adapted themselves in a complementary manner to reduce immune reactions and other pathogenic events in the parasite-host interface. It is known that ectoparasites produce a pharmacopoeia of bioactive molecules in their salivary glands which induce local immunomodulation of host responses consisting of a down-regulation of Th1 cytokines and up-regulation of Th2 cytokines leading to suppression of host antibody responses. These changes are linked to both successful blood feeding and pathogen transmission [100]. As a result, important functional molecules of ectoparasites that are exposed to the immune system of the host have likely low potency as immunogens and hence will be poor candidates for putative vaccines [101].

However, the discovery of the Bm86 protective antigen against *R. microplus* ticks revolutionized the vaccine development against ectoparasites [102–104]. Bm86 is a protein located at the epithelial cell membrane of the tick gut and consequently the host immune system is never in contact with this antigen. For this reason, Bm86 is named a concealed antigen. Tick attachment and feeding do not constitute a booster for these hidden antigens and repeated immunizations with the antigen will be necessary to keep high specific antibody titers against them. Though, they have the advantage over exposed antigens that ticks have not developed mechanisms to overcome the host immune response against them [105]. This vaccination approach requires that ectoparasites feed on host because the specific immunoglobulins against concealed antigen are taken up in the blood meal. For example, anti-tick effects of Bm86 based vaccines are produced by the interaction of the mediators of the host immune response ingested in the blood meal with the target antigen inside ticks. For these reasons, these vaccines against ectoparasites have no knockdown effects like the chemicals. Their effects are the same that those expressed in nature by species genetically resistant to ticks or with acquired resistance after

repeated tick infestations [106]. Increased mortality of ticks and eggs, decreased engorgement and egg mass weights, prolonged tick feeding and inhibition of molting are their effects whose overall result is the decreasing of the tick population after successive generations feeding on vaccinated animals.

Another drawback for immunological ectoparasite control is that parasite materials are often available in limited quantities, particularly in larval stages. Basic research in parasitology has provided some information on stage-specificity of host immune responses and potential sources of target antigens; however, immunoparasitologists are still faced with the problem of identifying individual antigens and recovering them in quantities sufficient for immunization trials. Genetic engineering technologies have given new hope to the search for anti-parasite vaccines overcoming many of these problems and providing significant breakthroughs in the development of these vaccines. Complete genome sequencing is becoming popular in identifying potential target genes for veterinary anti-parasite vaccines [107, 108] because it provides the necessary information for identification and functional analyses of the newly discovered genes. Molecular biology also makes possible the production and recovery of large quantities of antigens previously unattainable through conventional methods of biochemical isolation.

A successful general scheme for vaccine development involves the establishment of a suitable experimental model in order to define effector mechanisms of the immune response and the efficacy validation of protective antigens. In the case of the anti-ectoparasite vaccine development, infestation models are difficult to establish because the complexity of parasite life cycles. For ticks, there are attempts to use capillary feeding or artificial feeding on membranes in order to simplify the experimental models [109, 110], but the intricate mechanisms underlying interactions in the parasite- host interface that affect the efficacy of vaccine candidates have prevented the generalization of these models. Despite the inconvenience in the process of identifying protective immunogens, cloning the relevant gene(s), protein expression, purification process and demonstration of efficacy of the vaccine candidates against ectoparasites, it has been suggested that they represent about 10% of the final cost of vaccine development. Costs to convert promising vaccine candidates into commercial products may amount to six times the cost of vaccine development and production [111].

In 1998, a Bm86- based vaccine named Gavac™ was registered in Cuba [112, 113]. This vaccine was included in the National Program to control *R. microplus* ticks in cattle demonstrating an effective control of these ectoparasites under field conditions [114, 115]. This program was designed as a strategy of integrated management in which the Gavac™ vaccine is the main element

combined with a rational use of acaricides with proved effectivity. The acaricide baths are applied only when tick infestations are more than 10 adults per animal. The harmonic combination of these elements in the program allows the achievement of an economically acceptable tick level on the animal keeping the enzootic equilibrium for hemoparasites instead tick eradication. Strict discipline in the vaccine schedule application and not introducing unvaccinated animals into the herds are very important in order to ensure that ticks will always feed on a vaccinated animal. The reduction in the tick reproductive potential achieved by the anti-Bm86 antibodies warrantied obtaining a diminishing tick population after two or three generations.

In addition to these essential pillars of the Cuban program for cattle tick control, other management alternatives could be included in the local applications taking into account the specific context of each place where the program is applied such as the use of different cattle breeds, the employment of certain pastures that could inhibit tick survival or with improved nutrient value to enable cattle for the development of a better immune response after vaccination, the mowing of pasture, reduction of livestock density, the use of some biological controls among others. The universal character of this program is given by the wide possibilities for full adaptation of all these autochthonous practices in different regions. In this way, despite the "knockdown" effect is not a feature of this vaccine, the reduction in the use of chemicals with the consequent delay or elimination of resistant tick strains, useful life enlargement of these chemicals and diminution on food and environmental pollution are the most valuable effects of the vaccine's inclusion in the tick control programs that can be expected in the long term. After more than 20 years of the program application in Cuba, there is a significant reduction in the incidence of hemoparasitic diseases in the field [114, 116] because it was demonstrated that the vaccine not only diminished tick infestations but also diminished the tick vector capacity [117]. From an economical point of view, the overall effect obtained by Gavac™ vaccine application is a significant reduction in the cost of the ticks and tick borne diseases control [115].

A challenge for the implementation of this tick control strategy is that many livestock owners could not understand the basis of the program and considered it very complex, and time consuming. Correct program application implies knowledge about tick epidemiology, tick taxonomy, tick resistance, tick counting among others. Consequently, there is clearly a need for advisory technical support in order to supervise the compliance of intended procedures in the program, training of livestock owners and recording data periodically. All this experience in the immunological control obtained on livestock vaccination has demonstrated that the reduction of tick populations to acceptable levels is possible and that the

use of a unique control method is not enough to control complex parasites. It is evident that commercial companies and cattle producers, pet owners and public health services should assume the control of ectoparasites instead of attempting eradication. Governmental involvement and local well-organized regional policies with specialized technical support and careful monitoring of ectoparasite populations have also demonstrated to be essential in the most successful tick control programs implemented so far.

The success of tick control for companion animals or for the control of any other ectoparasites such as mosquitoes would need a more complex solution than that obtained for livestock. Pet owners would not desire to see any ticks on their animals, though one pathogen infected tick is enough to sicken the animal. The same occurs with human diseases transmitted by mosquitoes. A desirable vaccine for pets or humans should have an immediate effect on ectoparasites feeding on the host which will require a great deal of work by vaccine researchers to identify antigens with those effects.

9 Challenges in the Development of Fish Vaccines

Aquaculture has been the most promising sector for global fishery product with the increasing demand from consumers all over the world. The demand for fish and its products has gone up recently due to health benefits of a seafood diet. The global need for fish and fish products has increased since the last three decades. From 1990 to 2018, rise in total food fish consumption was up by 122%. It is interesting to note the importance of aquaculture as a main source of fish production in recent years. In 2018, global fish production from capture and aquaculture was 179 million tonnes slightly up from 2017 (173 million tonnes), with an estimated value of USD 401 billion, of which 82 million tonnes, valued at USD 250 billion, came from aquaculture production [118]. From 1990 to 2018, global capture fisheries production increased by only about 14% while global aquaculture production increased by 527%. In 2018, China remained the major aquaculture producer accounting for 35% of global aquaculture production while 34% from Asian countries excluding China [118]. Thus, it is inevitable to foresee the increasing global demand for the fish and fish products for human consumption and the importance of aquaculture as a major source.

Unfortunately, despite the successful story of fish and shrimp farming, there are some drawbacks. Infectious diseases are the most important concern in most intensive farming systems. Recently, due to adverse health concerns to the public, antibiotics and other chemicals are discouraged from being used to control infections in aquaculture farms [119]. Thus, there is a need to develop effective vaccine strategies in aquaculture to protect against infectious

diseases. Fish vaccine development is quite new compared to vaccines for livestock. Development of vaccines for salmonids, especially Atlantic salmon (*Salmo salar*), has been the best example of a successful program that resulted in better production and reduction of drug usage [120, 121]. It has been reported that 26 vaccines that have been licensed globally but this figure may be varied due to the scarce information from each country [122]. Fish vaccines are available only for bacterial and viral diseases while parasitic vaccines are also needed to control certain diseases. However, parasitic vaccine development may be quite a challenge due the antigenicity and mass production of the antigen. The administration of fish vaccines can be practiced by three different routes: injection, immersion, and oral administration. Vaccine administration by injection is the most popular method; the least being vaccination by oral route. As far as vaccine types are concerned, recent information shows that more than 70% are inactivated vaccine, followed by live-attenuated and other formulations (i.e., DNA and subunit vaccine). Research on fish mucosal immunity have shown that mucosal organs such as gills, skin, intestines and olfactory organs harbor lymphoid cells, including T and B cells as well as dendritic-like cells. Direct administration of antigens into the mucosal organs could facilitate development of fish mucosal vaccines [123].

Global demand for vaccine in aquaculture is increasing every year due to the continuing need for the routine usages of certain species such as salmonids and other species in the cold region. However, it is quite a challenge to develop vaccine for other fish species including carp, tilapia and catfish that are the major aquaculture fishes. Tilapias (*Oreochromis niloticus*) and striped catfish (*Pangasianodon hypophthalmus*) should be the next target of fish vaccine market since these species are popular all over the world with an annual production in 2018 at 4.7 and 4.3 million tonnes, respectively [118]. The main bacterial diseases of tilapias are caused by *Streptococcus agalactiae*, *Aeromonas* spp., *Francisella noatunensis* subsp. orientalis and *Flavobacterium columnare;* effective vaccines that are affordable will be helpful for aquaculture farmers [121]. Tilapia Lake Virus (TiLV) has been identified in many countries and vaccine trial is being conducted, however the impact of this viral infection may not be that high compared with the bacterial infections [124]. Vaccine for TiLV has been patented in USA, however, the commercial vaccine is not available. Reports on TiLV vaccine research in tilapia showed promising result [125], even though the number of studies has been limited. For striped catfish, commercial bacterial vaccine for *Edwardsiella ictaluri* and *Aeromonas* sp. are available [120, 122]. Strategic plans for successful vaccine development of these tropical species include the target antigens, route of administration, effective vaccine types and cost. In case of *S. agalactiae*, antigens and antigenic properties have been studied extensively that should lead to the proper and effective

vaccine design [126]. Genetic heterogeneity may be the main obstacle especially for conventional inactivated vaccine; thus, DNA and subunit vaccine are exciting strategies for many fish vaccine researchers to facilitate this problem. For example, multi-epitope vaccine of *S. agalactiae* has been designed and successfully developed for the protection against homologous serotype and cross immunity against heterologous serotype [127]. Design of vaccine against multivalent antigens is under investigated in tilapia which the outcome should be vital for the sustainability of the culture of this species. Route of administration relies on the fish farming methods of fish species. The most effective vaccine administration may not be practical with certain fish culture due to limitation of mass vaccination. Injection is the most effective vaccination method for fish but this method is practical only with suitable size and number of fish. Immersion should be the good choice for mass vaccination for fish culture that can be practiced with small size fish. Certain procedures have been developed to enhance the effectiveness of immersion vaccine such as nanovaccine which facilitates better adsorption on fish mucosal surfaces by certain nanoparticles mimicking the mucoadhesive characteristic of live pathogen [128]. Enhancement of fish skin mucosal immunity should be the target of future study since it is the first line of defense against the pathogens in the water environment. Oral vaccination is the least effective method due to antigen loss in fish digestive system. Certain procedures have been developed to protect the antigen from digestive enzymes including coating [129, 130]. Vaccine development and licensing are complicated processes resulting in high costs. High price of fish vaccines is the major impediment for global fish vaccine development. This is even more important for the low or middle price fish species that can barely absorb the extra costs in the production system. It is undoubtedly certain that fish production from aquaculture will be more important for the global consumption since production from capture fishery will decrease with time. Sustainable development of aquaculture must be facilitated with strong disease prevention programs that can be achieved by feasible vaccine strategy. Advanced vaccine strategies including DNA and mRNA vaccines should be developed that could be effective with the heterogenous bacteria and/or viruses. Cost of vaccine production is another challenge that should be solved by both the vaccine manufacturers and the stakeholders involved in aquaculture business.

10   Conclusion

The increase in global population has resulted in increase in the consumption and the use of animal products. Climate change has resulted in new and emerging diseases that is a major threat to global animal health and its welfare. Decreased animal productivity

could impact the global food security that would have adverse effect on the health of the human population. Low productivity would also affect the rural economies, especially the developing countries. Hence, there is a need to develop vaccines against pathogenic bacteria, virus, fungi, parasites and also to vectors like ticks that influence the health of animals. The current COVID-19 pandemic demonstrated the limitation of conventional vaccines. Hence new vaccination strategies should be developed to protect against animal diseases.

Overfishing has led to depletion of fish especially in warm waters. This has led to intense aquaculture in coastal areas. Industrialized aquaculture of fishes has led to the outbreak of diseases that could be addressed by use of vaccines. However, the challenge is to develop vaccines in the economy of scale that will benefit the stakeholders.

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Part II

Trends in Veterinary Vaccines

Chapter 2

Mammalian Cell Culture as a Platform for Veterinary Vaccines

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Abstract

For more than three decades, mammalian cells have been the host par excellence for the recombinant protein production for therapeutic purposes in humans. Due to the high cost of media and other supplies used for cell growth, initially this expression platform was only used for the production of proteins of pharmaceutical importance including antibodies. However, large biotechnological companies that used this platform continued research to improve its technical and economic feasibility. The main qualitative improvement was obtained when individual cells could be cultured in a liquid medium similar to bacteria and yeast cultures. Another important innovation for growing cells in suspension was the improvement in chemically defined media that does not contain macromolecules; they were cheaper to culture as any other microbial media. These scientific milestones have reduced the cost of mammalian cell culture and their use in obtaining proteins for veterinary use. The ease of working with mammalian cell culture has permitted the use of this expression platform to produce active pharmaceutic ingredients for veterinary vaccines. In this chapter, the protocol to obtain recombinant mammalian cell lines will be described.

Key words Mammalian cells, Cell culture, Suspension cell culture, Veterinary vaccines, Protein expression

1 Introduction

Proteins expressed by recombinant DNA technology offer many therapeutic advantages over traditional small molecule-based drugs; however, the need to use cells to obtain them, compared to the chemical synthesis, makes their production complicated. The continuous development of biochemistry, cell biology, molecular biology, and genetic engineering has prompted new discoveries that promise to guarantee these biopharmaceuticals in a safer, more efficient, and cheaper way [1].

There are numerous protein expression recombinant systems based on the use of microorganisms such as the *E. coli* bacteria [2] and yeasts [3], fungi such as *Aspergillus niger* [4], insect cells from

Spodoptera frugiperda and *Trichoplusia ni* [1, 5], mammalian cells [6, 7], plants [8], and transgenic animals [9]. The appropriate selection of the expression system takes into account several factors, among which the most important issues are related to the characteristics of the interested protein such as the number of disulfide bridges and posttranslational modifications, the use of the product, the safety and quality requirements for that use, scalability and of course, economic profitability [10]. Each expression system has its own advantages and limitations, but when complex proteins must be obtained, those based on mammalian cells are the most widely used due to their ability to carry out the correct protein folding and appropriate posttranslational modifications, such as glycosylation [11–13]. The mammalian cell culture is laborious and its productivity is low compared to that of microorganisms and furthermore was highly expensive at the beginning [14, 15]. Despite this, the high quality of the protein expressed compensated for these disadvantages [3, 16]. In addition, during the last three decades this system has undergone great improvements in order to meet the demands of the biopharmaceutical industry which has expanded its application scope [17].

Cell culture is the process by which the cells are cultivated in controlled conditions, generally out of their natural environment. The specific culture of animal cells can be described as *in vitro* maintenance and propagation of cells using an adequate nutrient medium. The most important and essential step in these cultures is the selection of adequate supplements to support the growth of this kind of cells [18]. There are two basic systems for the cell growth of superior organisms in culture, as monolayers in an artificial substrate (adherent or anchored culture) or floating in the culture medium (suspension culture) in which the individual or small aggregates from cells multiply while they are suspended in agitated liquid medium [19]. Most of the cell lines derived from vertebrates, with the exception of cellular hematopoietic lines were obtained as anchored cultures. However, currently many of these cell lines have been adapted to cultures in suspension [20, 21]. To achieve an ideal cellular suspension, the most common being a group of cells that grow fixed to a support are transferred to an agitated liquid medium where they are dispersed. After deleting the large cell aggregates, only the individual cells and small cell aggregates are transferred again to a fresh medium and after 2 or 3 weeks a suspension of active growth cells is produced. This cell suspension can then be propagated by regular subcultures of an aliquot of these cells to fresh medium. These suspension cultures can be handled like microbial cultures which eliminate many of disadvantages attributed to anchored cultures [22, 23]. In anchored or adherent cultures, cellular growth is limited by the surface area which limits the yields of product of interest when they are used as bioreactors. In suspension cultures, cellular growth is limited by the cell concentration in the medium and therefore the dilution of cellular

suspension with fresh medium is a way to stimulate growth and also allow the easy scale-up of these cultures. In addition, no enzymatic or mechanic dissociation of the cells is needed. Improvements in culture media and cellular adaptation to suspension growth have allowed successful cellular cultures in supplemented media with only small concentrations of serum or only with albumin, and in some cases in chemically defined media that does not contain macromolecules [24]. These media are cheaper and in consequence the spectrum of its application to the production of proteins with complex conformations that have application in the veterinary field is expanded [21, 25, 26].

Among mammalian cell lines commonly used in the production of therapeutic proteins are Chinese hamster ovary cells (CHO) [17], baby hamster kidney cells (BHK) [27], those derived from mouse myeloma NS0 [28] and SP2/0 [29] and also lines of human origin such as human embryonic kidney (HEK293) [30, 31]. A feature to highlight is that all these cell lines can grow in suspension cultures with serum-free media by using bioreactors and their production is easy to scale. Furthermore, they combine an easy-to-transfect system with the ability to secrete large amounts of proteins [32–34].

The development of a recombinant cell line expressing a foreign protein generally follows a common scheme of sequential steps and is ideally carried out in a chemically defined environment free of animal components [35]. After selecting the host cell line, the first step is the transfection of the cells with one or more expression vectors that contain the gene coding of interest protein and the selection marker [36]. This is followed by a selection phase, with the aim of enriching the set of cells that have integrated the transgene into their genome and that overexpress the protein of interest. This generates a heterogeneous cell population, in which each cell exhibits unique phenotypic and genetic characteristics [37]. Therefore, to ensure the monoclonality of protein-producing recombinant cell lines, one or more cloning steps are applied followed by screening based on high productivity [38]. At this point, if the whole process has been carried out in medium supplemented with serum, an adaptation phase of producer cells to serum-free, animal component-free or protein-free media should be included [39]. However, it is also possible to use host cell lines adapted prior to cultivation in serum-free medium and in suspension which reduces time and effort necessary to adapt them to these culture conditions [15, 40]. Subsequently, the characterization of the candidate clones is carried out in terms of cell growth in suspension, productivity (specific and volumetric), production stability, behavior at the shaken flask or bioreactor scale, and the quality of the generated product [36]. Detailed procedures to obtain mammalian cell lines expressing foreign proteins by using transfection with Polyethylenimine (PEI) and transduction with lentiviral vectors will be described in this chapter.

2   Materials

All materials and solutions are prepared using ultrapure water (18 MΩ-cm at 25 °C) and cell culture grade reagents. Solutions should be sterile-filtered through 0.2 μm filters.

2.1   PEI Preparation

PEI is a stable cationic polymer [41].

1. Dissolving the PEI (160,000 Da) (*see* **Note 1**) in water at 1 mg/mL. PEI would not fully be dissolved until the pH is adjusted to 7.0 by using 1 M HCl.
2. Sterilizing through a 0.2 μm filter, aliquot and store at −20 °C (*see* **Note 2**).

2.2   Glucose Preparation

1. Dissolve 5 g of glucose in 100 mL of water.
2. Sterilize through a 0.2 μm filter, aliquot and store at −20 °C.

2.3   Dulbecco's Modified Eagle's Medium (DMEM)

It can be obtained from any company (*see* **Note 3**).

2.4   Fetal Bovine Serum (FBS)

It can be obtained from any company (*see* **Note 4**).

2.5   CO$_2$ Incubator

It provides a stable environment designed to mimic a cell's natural environment with pH of 7.2–7.5, temperature of 37 °C, and a relative humidity of about 95%. The CO$_2$ concentration, about 5%, is controlled to match physiologic conditions and to maintain a constant pH.

2.6   Laminar Flow Cabinet

It is a carefully enclosed bench designed to prevent sample contamination (*see* **Note 5**).

2.7   Cell Culture Flasks, Dishes, and Plates

Cell culture flasks, dishes and plates must be specifically designed for successful growth and propagation of mammalian cells (*see* **Note 6**).

2.8   Inverted Microscopes

They are used for observing living cells at the bottom of a large container (e.g., a tissue culture flask) (*see* **Note 7**).

3   Methods

3.1   Transfection

It is a procedure that introduces foreign nucleic acids into cells to produce genetically modified cells either stably or transiently (*see* **Note 8**).

3.1.1 Transient Transfection of HEK293 Cell Line by Using PEI (See **Note 9**)

1. Seed HEK293 cells into a 6-well plate at a density of $\sim0.2 \times 10^6$ cells/mL using DMEM supplemented with 10% of FBS (*see* **Note 10**). Place in the incubator at 37 °C in a humidified 5% CO_2 atmosphere (*see* **Note 11**).

2. On the transfection day, 24 h after seeding, cells should be 75–80% confluent. Remove medium from the cells and wash carefully with PBS 1X (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4). Add 1.5 mL of DMEM without FBS and return the cells to the incubator (*see* **Note 12**).

3. Prepare separately sterile PEI and DNA in Eppendorf tubes. Mix thoroughly 10 μg of plasmid DNA (*see* **Note 13**) with 50 μL of 5% glucose and incubate at room temperature for 10 min. Mix thoroughly 10 μL of PEI + 50 μL of glucose 5% and incubate at room temperature for 10 min (*see* **Note 14**).

4. Place the mix PEI + glucose on the lid of the DNA + glucose Eppendorf and close it quickly so that PEI drops onto the DNA, mix thoroughly and incubate at room temperature for 10 min.

5. Add 900 mL of DMEM to the transfection cocktail (PEI + DNA) and mix gently.

6. Remove serum-free DMEM from cells and add carefully the transfection cocktail to each well of the culture plate (*see* **Note 15**).

7. Incubate the cells for 6 h at 37 °C in a 5% CO_2 humidified atmosphere.

8. Later add 1 mL of DMEM supplemented with 20% of SFB to each well so that the final concentration of SFB will be 10%. Incubate the plate overnight at 37 °C in a humidified 5% CO_2 atmosphere.

9. After 24 h remove the medium with the transfection cocktail from the cells and wash carefully with PBS 1×.

10. Add 3 mL of serum-free medium to each well and incubate the cells at 37 °C in a humidified 5% CO_2 atmosphere.

11. Collect medium samples from each well to analyze the expression of interest gene (*see* **Note 16**).

12. Evaluate expression of gene reporter (*see* **Note 17**).

3.1.2 Lentivirus-Mediated Transduction of HEK293 Cell Line

Lentivirus Production in HEK293-FT Cells

Lentivirus particles are obtained by cell transfection using the same PEI protocol described for transient transfection.

1. Twenty-four hours prior to transfection day, seed HEK293-FT cells using DMEM supplemented with 10% FBS. Incubate flasks at 37 °C in a humidified 5% CO_2 atmosphere (*see* **Note 18**).

2. On transfection day, the presence of a homogenous cell mono-layer with ~70–80% confluency should be observed using an inverted microscope. Remove metabolized medium, wash with 10 mL of PBS 1× and add 20 mL of DMEM medium if 175cm^2 T-flasks were used. Incubate flasks at 37 °C in a humidified 5% CO_2 atmosphere.

3. Prepare DNA for transfection in a sterile 50 mL centrifugation tube (*see* **Note 19**). For each 175 cm^2 T-flask, 70 μg of total DNA will be used. Taking into account that 10 μg of DNA are prepared in 50 μL of 5% glucose, for 70 μg of DNA per T-flask, means a total volume of 350 μL of 5% glucose. These quantities should be scaled up according to the flask number that will be transfected. Vortex for 1 min and incubate 10 min at room temperature.

4. Prepare PEI (160 kDa) for transfection in 1:1 of DNA/PEI ratio (w/w) as previously described. Add directly PEI mix to DNA transfection mix. Vortex for 1 min and incubate for 10 min at room temperature.

5. Top up the DNA/PEI mix with DMEM medium to a final volume of 3 mL/flask and add carefully this 3 mL of the DNA/PEI/DMEM mix to each 175 cm^2 T-flask containing the 20 mL of DMEM medium added previously. Gently tilt flask to cover all cells. Incubate flasks at 37 °C in a humidified 5% CO_2 atmosphere.

6. Six hours later, add FBS to a final concentration of 10%. Incubate flasks at 37 °C in a humidified 5% CO_2 atmosphere.

7. Forty two or 72 h after transfection, collect media from 175 cm^2 T-flasks into sterile 50 mL centrifugation tubes. This media contains lentivirus particles.

8. Centrifuge at ~500 × *g* for 5 min to pellet remaining cells and filter through a 0.45-μm membrane into a new sterile 50-mL centrifugation tubes (*see* **Note 20**).

9. The filtered viral supernatant can be stored at 4 °C for up to 3 days before concentration procedures, but it must be stored at −80 °C for longer periods. Minimize freeze-thaw cycles to avoid losses of virus titer.

Lentivirus Titration by Using an Assay Based on the HIV p24 Capsid Protein (*See* Note 21)

1. Concentrate lentivirus (*see* **Note 22**).

2. If Lenti-X™ Concentrator is used according to the manufacturer protocol, the lentivirus pellet is resuspended in 1/10 V of the original volume using DMEM medium. Aliquot in 1.5 mL sterile tubes and store at −80 °C. Minimize freeze-thaw cycles to avoid loss of virus titer.

3. Thaw one frozen aliquot of concentrated lentivirus and prepare serial dilutions in DMEM medium from 1/4000 to 1/256000 dilution.

4. Determine p24 capsid protein in the diluted samples using DAVIH-Ag P24 ELISA kit. Proceed with the assay as recommended by the manufacturer (*see* **Note 23**). Use DMEM medium as ELISA blank.

5. Calculate the amount of substance of p24 protein (n) in 1 mL of sample by dividing the mass of p24 protein obtained, expressed in grams, by its molecular weight (24×103 g/mol).

6. Calculate the number of p24 molecules in 1 mL of sample by multiplying the amount of substance calculated before by Avogadro's number (6×10^{23} molecules/mol).

7. Calculate the number of lentiviral particles in 1 mL of sample by dividing the number of p24 molecules per physical particle of lentivirus taking into account that there are around 2000 molecules of p24 protein per physical particle of lentivirus.

8. Finally, the number of infectious lentiviral particles in 1 mL of sample is calculated as 65% of total lentiviral particles [42].

Transduction of HEK293 Cell Line and Generation of Stable Recombinant Protein-Expressing Cell Pools in 24-Well Plates

1. Before lentiviral transduction of a cell line, its sensitivity to the drug used as selection marker in the transfer plasmid must be determined by a mortality curve vs. drug concentration according to the manufacturer protocol (*see* **Note 24**).

2. The best multiplicity of infection (MOI) for transduction of the specific cell line should be assayed (*see* **Note 25**). Accordingly, MOI, the volume of lentivirus stock for transduction is calculated as: Volume of lentivirus stock = (Needed number of infectious lentiviral particles)/(titer of lentivirus stock).

3. Seed the cells to be transduced at 2×10^4 cells per well in DMEM/F12 or DMEM medium supplemented with 5 or 10% of FBS (*see* **Note 26**). In the following steps, DMEM medium supplemented with 10% of FBS (DMEM+10% FBS) will be used for transduction and selection procedures. Incubate at 37 °C in a humidified 5% CO_2 atmosphere (*see* **Note 27**).

4. Sixteen to Twenty-four hours later, remove medium and wash cells with 1 mL of PBS 1× (*see* **Note 28**). Add 500 μL of DMEM medium to the control wells (control procedure, control+drug and control-drug). Add 500 μL of a mix of lentivirus stock and DMEM according to calculations on the **step 2**, to cell wells that will be transduced. Gently tilt the plate. To enhance the transduction efficiency, polybrene could be added to controls and cells to be transduced to a final concentration of 5–10 μg/mL (from 1 mg/mL stock solution) (*see*

Note 29). Incubate cells at 37 °C in a humidified 5% CO_2 atmosphere.

5. After 6 h, add 500 µL of DMEM supplemented with 20% SFB to control-drug. Moreover, add 500 µL of selective culture medium 2× (DMEM supplemented with 20% SFB and with 2× concentration of the appropriate selection drug) to control +drug and transduced cells. Incubate cells at 37 °C in a humidified 5% CO_2 atmosphere.

6. To increase the copy number integration of the interest gene, 16–24 h later, repeat **steps 4** and **5** for a second round of transduction.

7. Twenty-four hours after last round of transduction, gently aspirate the media and replace it with 1 mL of DMEM+10% FBS for control-drug cells and 1 mL of selective medium (DMEM+10% FBS + concentration of selection drug determined in **step 1**) to control+drug and transduced cells. Change the media every 48–72 h for approximately 14–21 days after selection drug was added the first time. Observe the percentage of surviving cells under an inverted microscope. Remember that control procedure cells will remain intact without any treatment or media exchange (Fig. 1).

8. After selection process, stable recombinant protein-expressing pool cells have been generated. At this point, collect media of control and transduced cells and replace with 1 mL of DMEM +10% FBS and check protein expression by the specific method designed according the protein nature (This method could be an ELISA, Dot Blot or Western Blotting among others).

3.2 Obtain Protein-Expressing Recombinant Clones by Limiting Dilution (See Note 30)

Limiting dilution cloning will be described here as selection method (*see* **Note 31**).

1. Once cell pools have been generated after 14–21 days under selective conditions in 24-well plate, collect cell culture media and detach adherent cells using 500 µL of trypsin-EDTA (Gibco or Sigma) at 37 °C in a humidified 5% CO_2 atmosphere for 3 min.

2. Gently dislodge the cell monolayer, transfer the cells to a sterile 50 mL centrifugation tube and quench the trypsin-EDTA solution using 5 mL of DMEM+10% FBS. As precaution, add fresh media to the 24-well plates and put them back in the incubator.

3. Centrifuge at 200 × g during 5 min and discard media.

4. Resuspend cell pellet in 1 mL of DMEM+10% FBS. Using a sterile serological 10-mL pipette, pipette up and down a few times, to break up any clumped cells. Check for cell aggregates (*see* **Note 32**).
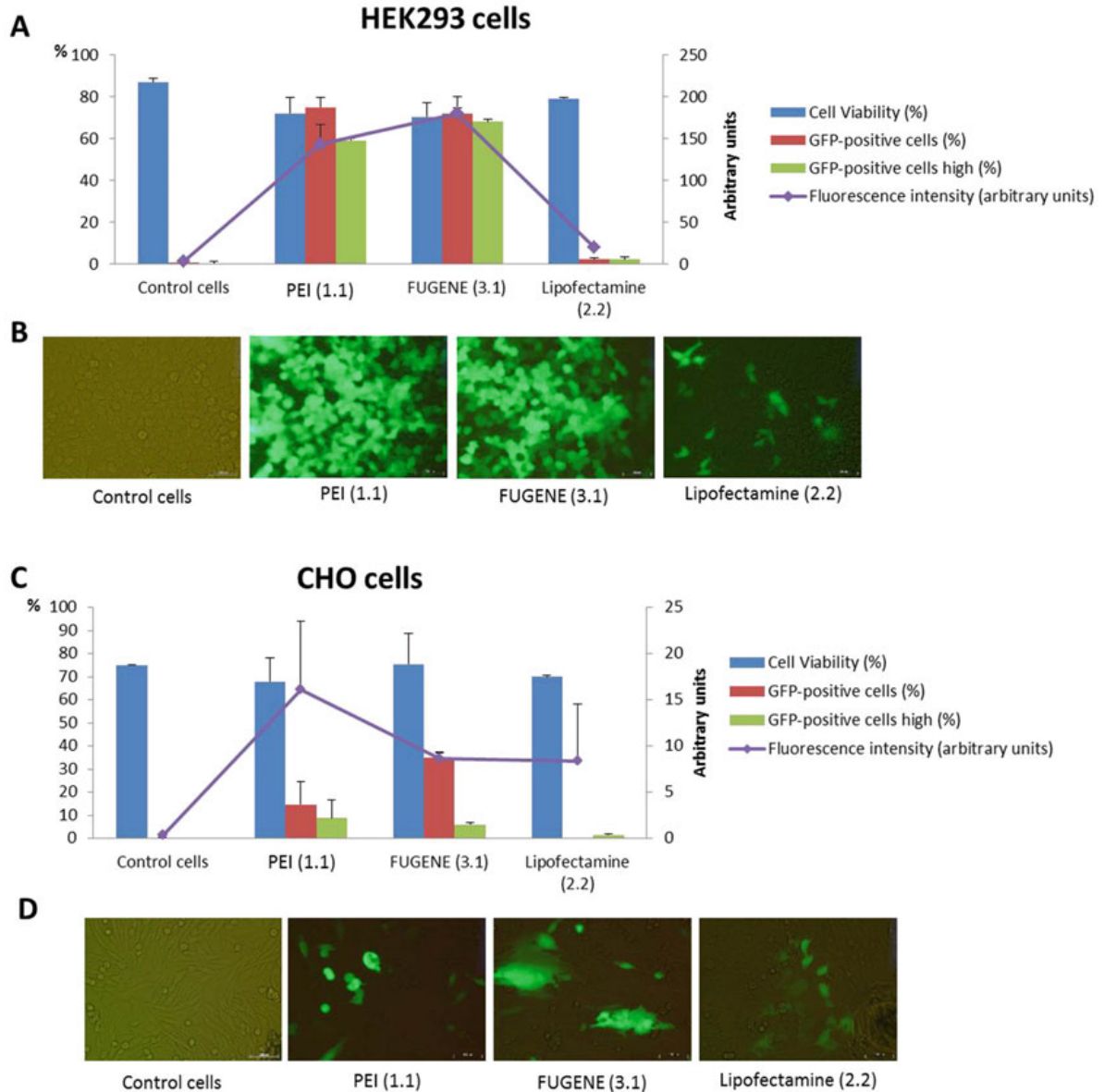
Fig. 1 Comparative analysis of transfection efficiency by flow cytometry and fluorescence microscopy in HEK293 and CHO cell lines, employing different transfection reagents. In FACS, fluorescence intensity of each sample was assessed using a PAS III flow cytometer (Partec-Sysmex) integrated with CellQuest software. The cells were analyzed with a 488 nm argon excitation laser and a 515–545 nm emission filter (FL1). Data analysis was performed using FloMax software, version 2.57. Cell samples were assayed at a medium flow rate until 30,000 cells. For fluorescence microscopy, images obtained by microscope (Olympus, USA) were processed with the ImageJ 1.48 software (NIH, USA). (**a** and **c**) Flow cytometry analysis and (**b** and **d**) fluorescence microscopy of HEK293 and CHO cell lines, respectively. Experiments were performed in triplicate. Mean values ± SD are shown

5. Determine cell density and viability using a hemocytometer and trypan blue exclusion.

6. In sterile 50 mL centrifugation tubes, perform a serial of cell dilutions in DMEM+10% FBS to obtain a final cell

concentration of 0.5 cells per 150 μL (*see* **Note 33**). For example, if the viable cell count in the **step 5** was 0.5×10^6 cells/mL,

Tube 1: To obtain 1000 cells/100 μL, dilute 200 μL of cells in 10 mL of DMEM+10% FBS.

Tube 2: To obtain 100 cells/100 μL, dilute 1 mL of cells from tube 1 in 9 mL of DMEM+10% FBS.

Tube 3: To obtain 10 cells/100 μL, dilute 1 mL of cells from tube 2 in 9 mL of DMEM+10% FBS.

Tube 4: To obtain 0.5 cells/150 μL, dilute 500 μL of cells from tube 3 in 15 mL of DMEM+10% FBS.

7. Transfer cell dilution from tube 4 to a sterile multichannel pipette tray. Fill each well of 96-well plates with 150 μL of this cell dilution using a multichannel pipette. Plate at least five 96-well plates to increase probability to pick high producing cells. Incubate the plates at 37 °C in a humidified 5% CO_2 atmosphere.

8. Seed remaining producing cells from **step 4** in 5–6 mL of DMEM+10% FBS using a 25 cm^2 T-flask. Incubate at 37 °C in a humidified 5% CO_2 atmosphere until 80% of confluency is reached and then expand cells to several 75 cm^2 T-flasks which will be seeded to 0.3×10^6 cells/mL in 12 mL of DMEM+10% FBS. When these last flasks have reached 80% of confluency and at least 90% of cell viability, cells could be cryopreserved in liquid nitrogen (see more details in Cryopreservation protocol).

9. Observe wells from plates seeded on **step 7** every day under an inverted microscope. After 10–15 days, colonies should start to appear (*see* **Note 34**). Only wells with a single colony should be taken into account for further analyses.

10. Collect cell culture media of selected wells from 96-well plates and replace with 150 μL of DMEM+10% FBS. Check protein expression by a specific assay for that protein like ELISA, western blot or Dot Blot.

11. Taking into account the protein expression level and cell colony size under microscope, pick up clones for expansion in 24-well plates containing 1 mL of DMEM+10% FBS (*see* **Note 35**). Detach cells of positive wells from the 96-well plate by gently pipetting the culture medium up and down with a micropipette. Transfer cells to a 24-well plate. Refill the selected wells from the 96-well plate with 150 μL of DMEM +10% FBS and repeat procedure as outlined above for detaching cells for 5 to 6 times. Incubate 24-well plates at 37 °C in a humidified 5% CO_2 atmosphere until around 80% confluency is reached.

12. Dislodge adherent cells as outlined above in **step 1**. Expand cells to 25 cm^2 T-flasks in 5–6 mL of DMEM+10% FBS. Incubate at 37 °C in a humidified 5% CO_2 atmosphere. As precaution, add fresh media to the 24-well plates and place them back in the incubator.

13. Detach cells from 25 cm^2 T-flasks using trypsin-EDTA procedure, and determine cell density and viability using a hemocytometer and trypan blue exclusion. For each clone to be tested for protein expression at this stage, transfer 1.2×10^6 cells, previously resuspended in fresh DMEM+10% FBS, into a sterile 15 mL centrifugation tube. Complete with medium to a final volume of 4 mL and gently resuspend the cells. Take 1 mL of this cell suspension and seed a well of a 24-well plate. Perform the experiment in triplicate for each clone. Incubate plates at 37 °C in a humidified 5% CO_2 atmosphere and 7–10 days later, collect and assay cell culture media for concentration of specific protein.

14. Seed remaining cells from each clone in the previous step at 0.3×10^6 cells/mL in 12 mL of DMEM+10% FBS in a 75 cm^2 T-flask. Incubate at 37 °C in a humidified 5% CO_2 atmosphere. When these last flasks have reached 80% of confluency and at least 90% of cell viability, cells could be cryopreserved in liquid nitrogen (see more details in Cryopreservation protocol). As precaution, add fresh media to the 25 cm^2 T-flasks and put them back in the incubator.

15. Taking into account the expression level of the protein of interest, select the five highest producing clones for adaptation to protein-free media and suspension culture.

3.3 Cell Adaptation to Chemically Defined Protein-Free Media and Suspension Culture (See Note 36)

A stepwise reduction of serum concentration from 10% to 2.5% combined with a gradual adaptation to a new chemically defined and protein-free medium (CDPFM) and a finally suspension culture will be described.

1. Before adaptation protocol starts, the growth rate and levels of specific protein expression of recombinant cell clones in the medium supplemented with 10% FBS and static culture must be well characterized (*see* **Note 37**).

2. Seed 0.3×10^6 cell/mL in 15 mL of DMEM+10% FBS in a 75 cm^2 T-flask. Incubate flask at 37 °C in a humidified 5% CO_2 atmosphere. Two-3 days later, cells should be 80–90% confluent.

3. Take a 1 mL sample of metabolized medium from 75 cm^2 T-flask, centrifuge at $500 \times g$ for 5 min and discard the pellet. Store cell culture media at −20 °C for further specific protein analyses.

4. Remove and discard the remaining metabolized medium, wash cells with 10 mL of PBS 1× and incubate them with 3–5 mL of trypsin-EDTA for 3–5 min at 37 °C in a humidified 5% CO_2 atmosphere.

5. Gently dislodge the cells and inactivate the trypsin-EDTA using 5–10 mL of DMEM+10% FBS medium. Pipette up and down a few times, using a sterile serological 10-mL pipette to break up any clumped cells (*see* **Note 38**).

6. Transfer suspension of detached cells into a sterile 50 mL centrifugation tube and centrifuge at $200 \times g$ for 5 min.

7. Discard media and resuspend the cells in 5 mL of DMEM+5% FBS (*see* **Note 39**). Take a sample to determine the cell density and viability using a hemocytometer and trypan blue exclusion.

8. According to the cell density, take the volume of cells needed to seed 0.5×10^6 cell/mL and complete to 15 mL of culture medium in a new 75 cm^2 T-flask. Incubate flask at 37 °C in a humidified 5% CO_2 atmosphere for 2–3 days.

9. As precaution, use remaining cell suspension to seed again the flask as in **step 4**. Add fresh DMEM+10% FBS medium to complete 15 mL and place it back in the incubator (*see* **Note 40**).

10. After 2–3 days, repeat **steps 3–9** (**step 9** is optional) for 3–6 times until cells show a recovery in DMEM+5% FBS with viability above 90% and similar doubling time (*see* **Note 41**).

11. Proceed to the next step in the adaptation process, with DMEM+2.5% FBS. Repeat **steps 3–10** for 3–6 times until cells show a recovery in DMEM+2.5% FBS with viability above 90% and similar doubling time.

12. Proceed to the next step in the adaptation process with a mix of 75% of DMEM+2.5% FBS and 25% of CDPFM (*see* **Note 42**).

13. Trypsinize cells previously adapted to DMEM+2.5% FBS, as described in **steps 4** and **5**. Resuspend the pellet of cells in 5 mL of the mix of 75% of DMEM+2.5% FBS and 25% of CDPFM. Take a sample to determine the cell density and viability using a hemocytometer and trypan blue exclusion. Cells should be in exponential growth phase and viability above 80%.

14. According to cell density, take the cell volume to seed 0.5×10^6 cell/mL and complete to 20 mL with 75% of DMEM+ 25% of CDPFM+ 2.5% FBS in a 125-mL Erlenmeyer flask (*see* **Note 43**).

15. Transfer the flask to an orbital shaker (80–120 rpm) placed in the incubator at 37 °C in a humidified 5% CO_2 atmosphere.

16. After 2–3 days, take 1 mL sample of suspension culture to determine the cell density and viability using a hemocytometer and trypan blue exclusion.

17. After cell counting, centrifuge the sample of the suspension culture at $500 \times g$ for 5 min and discard the pellet. Store cell culture media at $-20\ ^\circ$C for further specific protein analyses.

18. If there is a decrease in cell density and viability, transfer the entire cell suspension into a sterile 50 mL centrifugation tube and centrifuge at low velocity ($150 \times g$) during 5 min to discard death cells. Resuspend the pellet in a volume for seeding to $0.3–0.5 \times 10^6$ cell/mL in the mix of 75% of DMEM +2.5% FBS and 25% of CDPFM (*see* **Note 41**).

19. Transfer the flask on an orbital shaker (80–120 rpm) in the incubator at 37 $^\circ$C in a humidified 5% CO_2 atmosphere.

20. Repeat **steps 16–19** until cell density reaches $1–3 \times 10^6$ cells/ mL and viability above 90% in the mix of 75% of DMEM+2.5% FBS and 25% of CDPFM (*see* **Note 44**).

21. Repeat **steps 16–20** growing cells in a mix of 50% of DMEM +2.5% FBS and 50% of CDPFM. If the cells present a sustainable and reproducible growth for 3–6 passages, proceed to the next step.

22. Repeat **steps 16–20** growing cells in a mix of 25% of DMEM +2.5% FBS and 75% of CDPFM.

23. Repeat **steps 16–20** growing cells in a mix of 100% of CDPFM.

24. Amplify the culture in order to have enough cells for cryopreservation.

3.4 Cryopreservation Protocol

1. Count cells in exponential growth phase and viability over 90% and determine the volume needed for cryopreservation to a cell concentration of 10×10^6 cells/mL.

2. Centrifuge cells at $200 \times g$ for 5 min and break up the cell pellet by gently tapping the tube (*see* **Note 45**).

3. Prepare freeze medium. For cells cultured in serum supplemented media, use fresh culture medium with 8% or 10% (v/v) of DMSO and 20% (v/v) of FB. For cells adapted to CDPFM, use fresh culture medium or conditioned medium supplemented with 8% or 10% (v/v) of DMSO. Sterilize by filtration using a 0.22 μm filter. Prechill medium on ice before adding it on cells.

4. Add the appropriate volume of freezing medium to obtain desired cell concentration (i.e., Add 5 mL of freezing medium to 50×10^6 cells in order to freeze 5 vials with 10×10^6 cell/ mL each. Using a sterile serological pipette, gently mix cells during this process to keep a homogeneous cell suspension.

5. Quickly aliquot cells into the labeled vials (1 mL per vial) and immediately transfer the vials to a −80 °C freezer in a small styrofoam box or isopropanol-filled freezing container (*see* **Note 46**).

6. Twenty four to seventy-two hours later, transfer vials to storage in liquid nitrogen.

3.5 Growing Cell Clones in Suspension Culture in CDPFM

1. Seed 0.3×10^6 cells/mL in 250-mL Erlenmeyer flasks with a total CDPFM volume of 60 mL. Transfer flask to an orbital shaker set to 80–120 rpm in the incubator at 37 ° C in a humidified 5% CO_2 atmosphere.

2. After 2–3 days, count cells and transfer 54×10^6 cells into a sterile 50 mL centrifugation tube and centrifuge at $200 \times g$ for 5 min. Discard media and resuspend the cell pellet in 30 mL of fresh CDPFM.

3. Add 10 mL of this cell suspension to three 250-mL Erlenmeyer flask. Complete with fresh CDPFM to a total volume of 60 mL.

4. Transfer flasks to an orbital shaker set to 80–120 rpm in the incubator to 37 ° C in a humidified 5% CO_2 atmosphere.

5. Every 24 h, take 1 mL of each cell culture to determine cell density and viability by using a hemocytometer and trypan blue exclusion.

6. After cell counting, centrifuge the sample of suspension culture at $500 \times g$ for 5 min and discard the cell pellet. Store media at −20 °C for further specific protein analyses.

7. Determine growth rate, cell specific productivity and integrity of viable cell concentration by using following formulas:

   Growth rate (μ) in h-1 as $dVCD/dt = \mu \times VCD$ where VCD ($\times 10^6$ cells/mL) is viable cell density at time t in the exponential phase of cell growth.

   Specific productivity (QP) in pg/cell/day (pcd) in the exponential phase of cell growth as $(d[P])/dt = QP \times VCD$ where VCD ($\times 10^6$ cells/mL) is the integral of viable cell density and [P] is the concentration of the protein at time t (h).

   Integral of viable cell concentration (IVCC) at time t (10^6 cells×h/mL) as $IVCC(t2) = [(VCD(t2) + VCD(t1))]/2 \times (t2\text{-}t1) + IVCC(t1)$ where VCD ($\times 10^6$ cells/mL) is the viable cell density at time t (h).

8. Finally, select 2 or 3 clones with the best cell growth and productivity profiles in CDPFM and suspension culture to scale up and characterize in stirred bioreactors. Moreover, a

purification process and analytical and functional assessments of the expressed protein should be conducted (i.e., primary structure related to amino acid sequence and post-translational modifications, including glycans; higher-order structure; product-related substances and impurities, including size and charge variants; among others). Perform experiments *in vitro* and *in vivo* to study the biological activity of the protein.

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4   Notes

1. The most popular PEIs for transfection procedures are linear and branched ranging from 1 to 160 kDa. There is a positive correlation between transfection efficiency and cell cytotoxicity with the PEI molecular weight (The highest PEI molecular weight, the highest transfection efficiency and cell cytotoxicity).

2. Newly prepared batches of PEI should be titrated by setting up a transient transfection with a control plasmid expressing an easily detectable reporter protein such as Green Fluorescent Protein (GFP). Transfection efficiencies can be determined after 48 h by calculating the percentage of GFP-positive cells using flow cytometry or a fluorescence microscope. PEI can be stable for 6 months at $-20\ °C$. Make small aliquots depending on how much is needed.

3. DMEM contains four times the concentration of amino acids and vitamins than the original Eagle's Minimal Essential Medium. It is originally formulated with low glucose (1 g/L) and sodium pyruvate, but is often used with higher glucose levels and contains no proteins, lipids, or growth factors. It uses sodium bicarbonate buffer system (3.7 g/L), and therefore requires a 5–10% of CO_2 environment to maintain physiological pH. Commonly, it must be supplemented with 10% Fetal Bovine Serum (FBS).

4. FBS comes from the blood drawn from a bovine fetus. It is the most widely used serum-supplement for the in vitro cell culture of eukaryotic cells due to its content of growth factors. FBS is not a fully defined media component, and as such may vary in composition between batches. Depending on the origin, it could contain specific bovine viruses or infectious agents. It is advisable to buy virus and mycoplasm tested FBS and to test its batches for cell toxicity.

5. Air is drawn through a HEPA filter and blown in a very smooth, laminar flow toward the user. There are many different types of cabinets with a variety of airflow patterns in both horizontal and vertical configurations. Vertical laminar flow is strongly

recommended to work with mammalian cell lines. Laminar flow cabinets may have a UV-C germicidal lamp which is usually kept on for 15 min to sterilize the interior before usage.

6. The tissue culture treatment process involves exposing a polystyrene microplate to a plasma gas in order to modify the hydrophobic plastic surface to make it more hydrophilic. Most common varieties include flat-sided tissue culture flasks, Erlenmeyer flasks, spinner flasks, dishes of different diameters and culture plates of 6, 12, 24 and 96 wells.

7. Their light source and condenser are placed on the top and the objectives are placed below the sample. There is no contact between objective and sample and sterile working conditions are possible. These microscopes may also be fitted with accessories for fitting still and video cameras, fluorescence illumination and many other applications.

8. The choice of stable or transient transfection depends on the objective of the experiment. The integration into the host genome of genetic materials used for transfection is the main characteristic of a stable transfection. A marker gene for the selection of recombinant cells is generally incorporated to the vector that is used for this kind of cell transfection and transgene expression is sustained even after host cell replication. In contrast, transiently transfected genes are only expressed for a limited period of time and are not integrated into the genome. DNA can be introduced into a host cell by transfection with different methods such as mediated by calcium phosphate, cationic polymer, ultrasound, electroporation, virus-mediated among others [14]. Once a cell line is chosen, the best transfection protocol on experimental conditions should be established (Fig. 2). All culture and transfection procedures are carried out in a laminar flow cabinet.

9. PEI condenses DNA into positively charged particles, which bind to the anionic cell surface. Consequently, the DNA/PEI complex is endocytosed by the cells and the DNA released into the cell cytoplasm [43]. This protocol is appropriate for successful transfection of many different mammalian cell lines. In our laboratory, it was determined experimentally the best protocol for transfection of HEK293 cells.

10. Cell growth media and PBS 1X should be warmed to 37 °C prior to the contact with cells.

11. Cells that will be used for transfection must have more than five passages after defrosting from liquid nitrogen and they must be in exponential growth. In our experience, a ~70–80% of cell confluency at the transfection moment is crucial for a successful cell transfection. Confluencies lesser than 70% are low cell densities that could negatively affect the yield of expressed
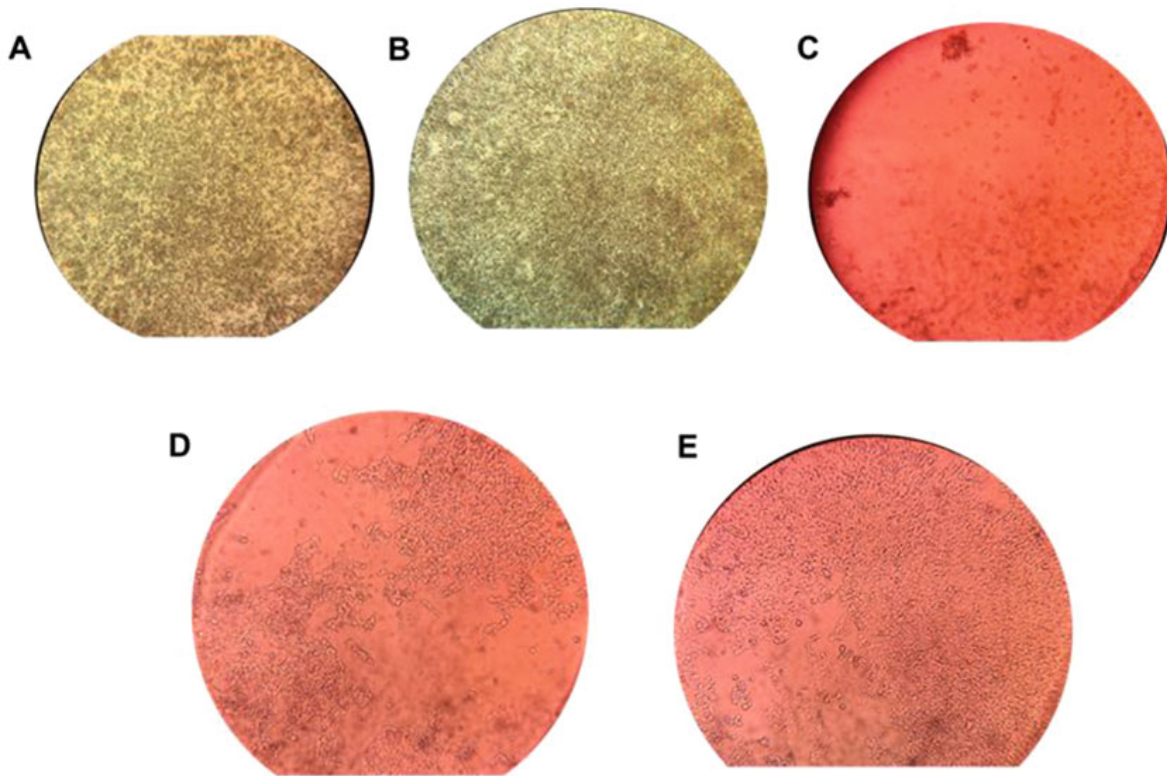
Fig. 2 Generation of stable protein-expressing recombinant HEK293 cells after 10 days under blasticidin selection. (**a**) Control of procedure: cells that were not transduced neither undergone the transduction and selection procedures. They remained intact during the transduction and selection periods. (**b**) Control-drug: cells that were not transduced but undergone the transduction and selection procedures but were cultured without selection drug. (**c**) Control+drug: cells that were not transduced but undergone the transduction and selection procedures and were cultured with selection drug. (**d**) Cells transduced at a MOI of 50 with lentivirus bearing the interest gene and blasticidin resistance gene. (**e**) Cells transduced at a MOI of 100 with lentivirus bearing the interest gene and blasticidin resistance gene

proteins. Confluencies higher than 80% could cause the detachment of cell monolayer during transfection procedures and could also negatively affect the yield of expressed proteins.

12. FBS might interfere with the transfection process and decrease transfection efficiencies. Therefore this step is important in order to adapt cells to serum-free medium.

13. High-quality DNA must be prepared for transfection purpose. After DNA precipitation, a final sterilizing wash with 70% ethanol will be performed. DNA will be resuspended in sterile water.

14. Transfection efficiencies can vary according DNA/PEI ratios. The protocol outlined here uses a 1:1 ratio of PEI/DNA (w/w) because it was found to be optimal for genes expressed by our group. However, this ratio should be optimized for each gene to be expressed. Ratios between 1:1 and 4:1 could be routinely screened.

15. A transfection negative control should be included in which cells are transfected with the empty vector without the coding sequence of the interest protein. In addition, reporters could be also included as positive control to measure transfection efficiency. Reporters are called to those genes expressing easily identified and measured proteins or selectable markers. They are often used as an indication of insertion or expression of another gene of interest in transfected cells. Due to the variability of the transfection efficiency, experiments must be performed with at least three replicates by assayed condition.

16. Samples of culture media from each well should be taken between 48 and 72 h post-transfection in order to check expression of the foreign protein. The best time to quantify the protein expression should be optimized previously. The presence of this protein can be measured by western blot, ELISA or any specific biological assay designed to do that.

17. For fluorescent gene reporters as GFP, expression analysis by Fluorescence-Activated Cell Sorting (FACS) and/or Fluorescence Microscopy could be carried out. Fluorescence intensity data can be used to generate graphs indicating relative protein expression respect to GFP (Fig. 1).

18. Five T-flasks of $175 cm^2$ are recommended for this transfection procedure in order to obtain high lentivirus titers. However, the procedure can be scaled-down to $75 cm^2$ T-flasks. HEK293-FT cells can be cultured in DMEM or DMEM/F12 supplemented with 5% or 10% of FBS. Seeding densities must be adjusted to reach ~70–80% confluency at the moment of transfection as described for PEI transfection protocol and in the **Note 10**. It should be taken into account that different cells will have different growth rates depending on culture media and percentage of supplemented FBS.

19. Third generation HIV-1-based LV packaging system (Invitrogen) is recommended. This system is based on an interest gene encoding transfer plasmid and three helper plasmids: pLP1 (encodes the viral gag and pol genes), pLP2 (encodes the viral rev gene) and pLP-VSVG (encodes the envelope G glycoprotein from Vesicular Stomatitis Virus). For each $175 cm^2$ T-flask, 70 μg of total DNA will be used in 2:1:1:1 (w/w/w/w) transfer/pLP1/pLP2/pLP-VSVG plasmid ratio in 5% of glucose in a final volume of 350 μL.

20. Do not use a 0.2 μm filter because this may shear the viral particles. Use only cellulose acetate or polyethersulfone (PES) (low-protein-binding) filters. Avoid the use of nitrocellulose filters because it binds proteins on the lentiviral envelope and destroys the virus particles.

21. Several approaches have been described for titration of lentivirus that will allow a better adjustment of the infection multiplicity. Assays for titration include (a) quantitative reverse transcription polymerase chain reaction (qRT-PCR) to amplify lentiviral RNA, (b) qPCR to amplify proviral DNA from genomic DNA of transduced cells, (c) detection of the HIV p24 capsid protein, (d) flow cytometry for lentivirus' preparations holding a fluorophore gene, and (e) colony forming assay for lentivirus preparations that confer antibiotic resistance [44–46]. Herein, DAVIH-Ag P24 ELISA kit was selected for titration of lentivirus stocks. This is an assay based on the HIV p24 capsid protein quantification [47, 48].

22. There are several options to concentrate lentivirus such as ultracentrifugation, anion exchange chromatography and precipitation using PEG 6000. Lenti-X™ Concentrator (Takara, Clontech) is recommended because it is a fast and simple method [44].

23. This commercial p24 ELISA kit contains inactivated wild-type HIV-1, which is potentially infectious and represents a safety risk. Therefore, this assay should be performed using Biosafety Level 2 (BL2) conditions.

24. The drug concentration required for selection of resistant mammalian cells will depend on the cell line, the promoter driving expression of the selection marker's gene and the culture medium. It is recommended to perform selection process in culture medium supplemented with 5% or 10% of FBS.

25. The multiplicity of infection (MOI) is defined as the number of infectious virus particles that is applied per target cell at the time of infection [46]. Testing MOI values from 5 to 100 are recommended for transduction of cells such as HEK293. For hard transduce cells such as CHO, use MOI values from 100 to 800. Try different MOI values in the same experiment. The number of infected cells by the number of lentiviral particles is calculated as previously reported [46].

26. For transduction, selection and cloning procedures, cells can be cultured in DMEM/F12 or DMEM medium, or a specific culture medium for a cell line, supplemented with 5% or 10% FBS. For cells pre-adapted to protein-free media and suspension culture, we recommend to culture cells with 5% FBS to speed re-adaptation to anchored culture conditions. It is also recommended to include wells of cells to be used as controls of transduction and selection procedures.

27. The number of seeded wells will depend on the different MOIs to be used and controls that should be included such as (1) cells that will not be transduced and cultured without selection drug (control-drug: This control will be monitored to determine

how experimental procedures such as cytotoxicity of culture medium, washing steps, incubation for several hours in a medium without SFB will affect cells), (2) cells that will not be transduced but will be cultured with selection drug (control +drug: This control will allow to determine the time when all non-transduced cells have died by incubation with the selection drug) (3) cells that will remain intact during the experiment (control procedure: This control will be monitored to determine cell growth kinetics under normal culture conditions and how transduction procedure affects cells) and for 24-well plates, reserve 6 wells to perform the mortality curve (cells without transduction exposed to increasing drug concentrations).

28. At this stage, under microscope, an adherent and homogeneous monolayer should be observed in each well.

29. Polybrene (hexadimethrine-bromide) is a cationic polymer that can greatly enhance the efficiency of retroviral or lentiviral infection to the mammalian cells. It acts by neutralizing the charge repulsion between virions and the cell surface, thus increasing infection efficiency from 100 to 1000 fold. The optimal concentration of polybrene should be determined for each cell line. For CHO and HEK293 cells, transduction can be performed in the presence of a final concentration from 8 to 10 μg/mL of polybrene. It is important the inclusion of control cells in this treatment since polybrene could influence the cell morphology.

30. Lentiviral vectors allows gene integration into transcriptionally open chromatin in the transduced cells, therefore it is a highly efficient method for stable transgene expression [46]. However, the integration event is random and independent in each transduced cell, which leads to a transduced pool of cells with different chromosomal integration sites, copy-numbers inserted and heterogeneous protein expression levels [49]. Additionally, low producer cells of foreign protein use their energy mainly to growth resulting in making up that they are the majority inside population. Therefore, cloning and selection procedures must be employed to isolate the highest producer individual cells and obtaining a homogeneous population of cells from a single cell. There are several approaches, from manual methods to complex automated and high-throughput cell screening technologies [49, 50]. Although, classical methods of clone isolation such as limiting dilution are highly labor intensive and low-throughput technologies, they are preferred due to their low cost, ease of implementation and lack of specialized equipment required. In order to ensure monoclonality, multiple rounds of subcloning must generally be performed using the selected clones. Despite it is statistically

possible ($p > 0.99$) to get a monoclonal culture after two rounds of limiting dilution cloning, the phenomenon known as the persistence of mixed clones could prevent this monoclonality [50].

31. For a detailed description of other clone selection and screening procedures other literature should be reviewed [49–52].

32. Aggregates reduce clonal probability of emerging colonies when limiting dilution is performed.

33. This limiting dilution cloning can be performed directly from the entire cell pool or dividing it into smaller pools (minipools) in order to isolate clones from the highest producing minipools. This last approach will increase the probability to pick high producing cells from a heterogeneous population [53].

34. The time in which colonies start to appear depends on the cell line and culture media composition. For instance, colonies from CHO cells start to appear after 8–10 days, meanwhile, colonies from HEK293 cells, appear after 15 days.

35. When clones are selected by using limiting dilution procedure, two criteria should be taking into account: colony size and expression level of interest protein. Both of them are properties very important for further stable cell line establishment. Colony size is related to growth rate and the expression level to a specific productivity. Medium-sized colonies and stronger ELISA or Dot Blot signal are preferred due to they could have a good growth rate and a high specific productivity. Meanwhile, small-sized colonies with high level of protein expression could indicate high specific productivity but usually also have a poor growth rate [53].

36. In spite of growth-promoting advantages of serum as a rich source of nutrients, growth factors, hormones, protective elements, attachments factors, among others; its addition to culture media has also disadvantages derived of its batch-to-batch variation and its undefined composition. These last serum characteristics could lead to inconsistent growth and productivity; high protein content, mainly albumin that hampers purification of the final product and potential contamination with adventitious agents such as viruses, mycoplasma and prions. In addition, ethical concerns and from the economic point of view the availability and high cost are also serum drawbacks [32]. Therefore, the removal of serum as a supplement in the biopharmaceutical setting is highly recommended by regulatory agencies. Use chemically defined serum-free or protein-free media that offer lot-to-lot consistency, avoid potential contamination with adventitious agents and decrease

production costs; while maintaining optimal cell growth, viability and productivity. Moreover, suspension systems instead of adherent culture are preferred because of easier culture procedures, upstream/downstream processing and scale-up [54]. Therefore, the adaptation of recombinant cell lines to chemically defined and protein-free media and suspension culture is necessary to meet economical and regulatory demands. There are several approaches to adapt cells to those culture conditions: direct adaptation, sequential adaptation, reduction of serum content and suspension adaptation [54, 55]. These processes are difficult, time-consuming, stressful for the cells, and should be done individually for each cell line and production process [55]. Furthermore, the recombinant cell lines should be closely monitored throughout the adaptation process, because depending on the product or cell type, the productivity of the recombinant cell line can be lower or higher after this process [56] and glycosylation patterns could change the biological activity and/or in vivo protein half-life [57–59]. However, the use of pre-adapted to chemically defined and protein-free media and suspension culture host cell lines, greatly reduces or eliminates the need for further adaptation of resulting recombinant cell lines to these culture conditions [15, 24]. In that case, cell line transfection and clone selection process must be performed in suspension culture for which there is less experience because these are more recent developments.

37. In order to characterize the growth rate, the population doubling time must be calculated using the following equation: $Dt = t \times \log2/[\log(N/No)]$ where t is cultivation time, N is the final number of cells, and No is the initial number of cells [55]. Cells should be in exponential growth phase and viability above 90% for beginning the adaptation protocol. Meanwhile, the specific protein expression level of cell clones must be determined in an assay on 24-well plate by using the procedure described on **step 13** of the Subheading: Obtaining protein-expressing recombinant clones by limiting dilution.

38. When cells are cultured in media with lower serum concentration than 10%, less quantity of trypsin-EDTA solution is required to detach adherent cells.

39. The cell pellet should be resuspended in the culture medium with the same serum concentration in which cells will be adapted to growth. If cells should be cultured in DMEM+5% FBS or DMEM+2.5% FBS, resuspend the cell pellet in 5 mL of DMEM+5% FBS or DMEM+2.5% FBS, respectively.

40. After passing cells to a culture with different conditions; preserve cells growing in the previous condition in order to avoid

starting from the beginning of the adaptation process just in case cells are damage in the new growth conditions.

41. If there are not enough cells to seed a flask at 0.5×10^6 cells/ mL in 20 mL, decrease the total volume keeping this cell concentration. If it is necessary, use smaller size flasks.

42. Adaptation to suspension culture can be performed at the end of chemically defined and protein-free adaptation. However, to save time and resources, starting at this point culturing cells in shaken culture is recommended. Some cell lines require separate serum-free and suspension adaptation steps. If the cells died abruptly in the adaptation to lower serum concentrations and suspension culture; firstly, try to adapt the cells to lower serum concentrations and CDPFM in static culture and after, proceed with cell adaptation to suspension culture. Lower agitation velocities or spinner flasks can be used to reduce suspension growth stress to cells.

43. Erlenmeyer flasks should be used for growing suspension cells in a shaker. If they are not available $25cm^2$ or $75cm^2$ T-flasks could be also used. When $25cm^2$ T-flasks are used in vertical position in shaken culture, only 10 mL of total volume should be used to avoid medium spillage through the semi-opened cap. If $75cm^2$ T-flasks are used in vertical or horizontal position in shaken culture, a maximum medium volume of 20 mL should be used to avoid the medium spillage through the semi-opened cap.

44. If cells show a sustainable and reproducible growth for 3–6 passages, achieving $1\text{-}3 \times 10^6$ cells/mL and cell viability above 90%, adaptation to this growth conditions should be considered and it is possible to proceed to the next step in the adaptation process

45. Conditioned medium is the metabolized medium obtained after the cell suspension has been centrifuged. This medium is filtered through a 0.22 μm membrane into a sterile 50 mL centrifugation tube and used to prepare the freezing medium.

46. If styrofoam box or isopropanol-filled freezing container are not available, aliquot cells into vials and transfer to ice. Later, introduce the vials into a small size nylon bag, wrap it in a piece of cloth or cotton and, immediately transfer to a -80 °C freezer.

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Chapter 3

Avian Paramyxoviruses as Vectors for Vaccine Development

Shin-Hee Kim, Edris Shirvani, and Siba Samal

Abstract

Avian paramyxoviruses (APMVs) have gained a great attention to be developed as vaccine vectors against human and veterinary pathogens. Avirulent APMVs are highly safe to be used as vaccine vectors for avian and non-avian species. APMV vectored vaccines induce robust cellular and humoral immune responses in a broad range of hosts. APMV vectors can be a good platform by facilitating rapid generation of vaccines against emerging pathogens. In this chapter, we discuss application of reverse genetics of APMVs for vaccine development, design of APMV vectored vaccines, cloning of protective antigen(s) into a vector, recovery of vectored vaccines and characterization of generated vaccine viruses.

Key words Avian paramyxoviruses, Viral vector, Vaccine development, Human vaccines, Veterinary vaccines, Protective antigens, Reverse genetics

1 Introduction

Vaccination is one of the most effective control measures for human and veterinary infectious diseases. However, there are many human and animal diseases for which vaccines are not available or the available vaccines do not provide adequate protection. In particular, the majority of the veterinary vaccines are either inactivated or live-attenuated [1]. The inactivated vaccines usually are not cost-effective for veterinary use and do not provide long-term immunity. The live-attenuated vaccines have the tendency to revert back to virulence. Therefore, there is a great need to develop improved vaccines against existing and emerging human and veterinary pathogens.

Replicating viral vector vaccines offer a live virus vaccine approach without requiring involvement of the complete pathogens and can induce protective humoral and cellular immune responses [2]. A variety of DNA and RNA virus vectors are currently available for human and veterinary vaccine development. Among these vectors, APMVs have several characteristics suitable

for the development of human and veterinary vaccines. APMVs belong to the family *Paramyxoviridae* and the genus *Avulavirus* [3]. APMVs have nonsegmented, single-stranded, negative-sense RNA genome containing 6 genes in the order of 3-N-P-M-F-HN-L-5. Each transcriptional unit contains a major open reading frame flanked by short 5′ and 3′ untranslated regions (UTRs), which are followed by conserved transcriptional initiation and termination control sequences, known as gene-start (GS) and gene-end (GE), respectively. The genome length of APMVs must be an even multiple of six for efficient virus replication following the "rule of six." Among APMVs, avian paramyxovirus serotype-1 (Newcastle disease virus, NDV) is the best characterized vector with a proven track record of safety and efficacy [4, 5]. NDV and other serotypes of APMVs have several advantages as vaccine vectors. First, all non-avian animal species do not have pre-existing antibodies to APMVs. Second, APMVs are highly safe in non-avian species due to natural host range restrictions. Third, particularly, NDV has a wide host range; therefore, can be used as a vaccine vector for most animal species. Specifically, NDV-vectored vaccines have been evaluated in several animal species (i.e., chicken, cattle, sheep, cat, mouse, pig, and dog) for veterinary use and non-human primates for human use [6]. Lastly, APMVs do not recombine nor integrate into host cell DNA. Therefore, they are highly safe as vaccine vectors.

Reverse genetics has been widely used to generate infectious viruses entirely from cloned.

cDNA [7]. Infectious APMV can be recovered by transfecting cultured cells with plasmids encoding the viral full-length antigenomic RNA and ribonucleoproteins (N, P, and L proteins) involved in replication and transcription under the control of bacteriophage T7 RNA polymerase promoter (Fig. 1). This reverse genetics technique system has been available for various NDV strains and other APMV serotypes [8–13]. Furthermore, APMVs have shown to accommodate insertion of a foreign gene as an additional transcriptional unit, thus facilitating APMVs to be used as vaccine vectors against human and veterinary pathogens [7, 12]. Further, APMV vectors can be used as a platform for rapidly developing vaccines against emerging pathogens by identifying their protective antigen (s) [14]. For the veterinary vaccine development, protective efficacy of avirulent NDV (i.e., LaSota and B1), chimeric NDV, and avian paramyxovirus serotype-3 (APMV-3) vectored vaccines have been evaluated and verified by many different vaccination studies [5, 11–13, 15]. All these different APMV vectored vaccines can be generated by using a universal approach. Therefore, we have illustrated a standardized protocol for construction and recovery of APMV vectored vaccines and characterization of the generated vaccine viruses.
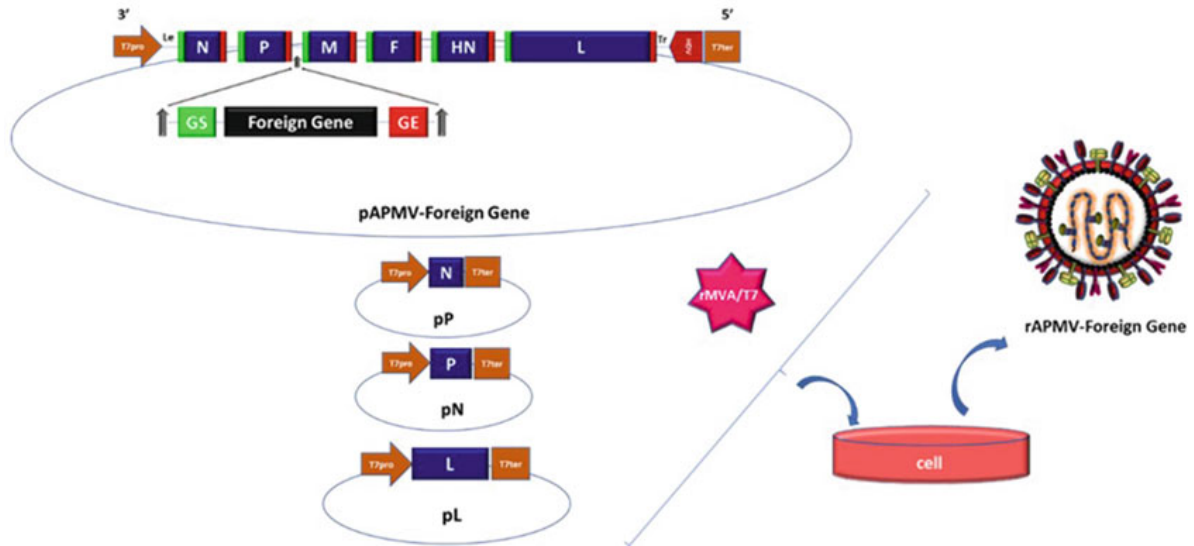
Fig. 1 Schematic diagram for generation of recombinant APMV expressing a foreign protein. Infectious APMV can be recovered by transfecting HEp-2 cells with plasmids encoding the viral components of full-length antigenomic RNA and ribonucleoprotein (the N, P, and L proteins) under the control of bacteriophage T7 RNA polymerase promoter. The T7 RNA polymerase is provided by the recombinant vaccinia MVA-T7

2   Materials

2.1   Preparation of a Foreign Gene (Insert) for Cloning

Synthesized foreign gene.

Platinum Taq DNA polymerase.

Primer set (forward and reverse, 10 µM stock concentration; **Note 1**).

dNTPs (dATP, dCTP, dGTP, and dTTP, 10 mM; **Note 2**).

Agarose-TAE gel (1%; **Note 3**).

6× DNA loading dye.

Tris-acetate-EDTA (TAE) electrophoresis buffer (*see* **Note 4**).

1-kb plus DNA ladder.

DNA purification kit.

Restriction enzyme.

Midi plasmid purification kit.

Mini plasmid preparation kit.

Alkaline phosphatase.

Ligase.

E. coli (DH10B) chemically competent cells.

Ice.

Low-salt LB broth and agar plate supplemented with 5 µg/ml tetracycline (*see* **Notes 5** and **6**).

PCR tubes (0.2 ml).

Thermal cycler.

UV transilluminator.

Razor blades.

1.5-ml microcentrifuge tubes.

Spectrophotometer (e.g., NanoDrop, Thermo Fisher Scientific).

Water bath (42 °C).

Incubator (30 °C).

Rocking incubator (30 °C).

Sterile 15-ml round-bottom culture tubes.

2.2 Transfection

Human epidermoid carcinoma cell line (HEp-2).

Plasmids: full-length AMPV containing a foreign gene and three support plasmids (pTM1-N, pTM1-P, and pTM1-L).

Opti-MEM medium.

Transfection agent.

Modified vaccinia virus Ankara (MVA-T7; BEI Resources, cat. no. NR-1).

Phosphate-buffered saline (PBS; **Note 7**).

Dulbecco's Modified Eagle Medium (DMEM).

Penicillin/streptomycin solution (100×).

Freshly collected allantoic fluid from 10- to 11-day-old embryonated chicken eggs (store up to 1 week at 4 °C).

9- to 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (Charles River).

Chicken embryo fibroblast cell line (DF1).

70% ethanol.

1% chicken red blood cells (RBC).

Methylcellulose medium (*see* **Note 8**).

Fetal bovine serum (FBS).

Methanol.

1% crystal violet.

Biosafety cabinet class II.

6-well tissue culture plate.

37 °C, 5% CO_2 humidified incubator.

Disposable spoon.

Conical centrifuge tube (15 ml).

Centrifuge.

V-bottom 96-well plates.

Vortex mixer.

Micropipette tips, sterile.

1.5-ml microcentrifuge tubes

3 Methods

3.1 Construction of Full-Length APMV Genome Containing the Gene of a Protective Antigen

In general, a foreign gene flanked by APMV gene-start (GS) and gene-end (GE) sequences is inserted into a 3 noncoding region of an APMV genome as an additional transcription unit [7]. The induction of robust immune response requires high levels of antigen expression. For instances, this can be achieved by a codon-optimization of protective antigen gene sequence and by
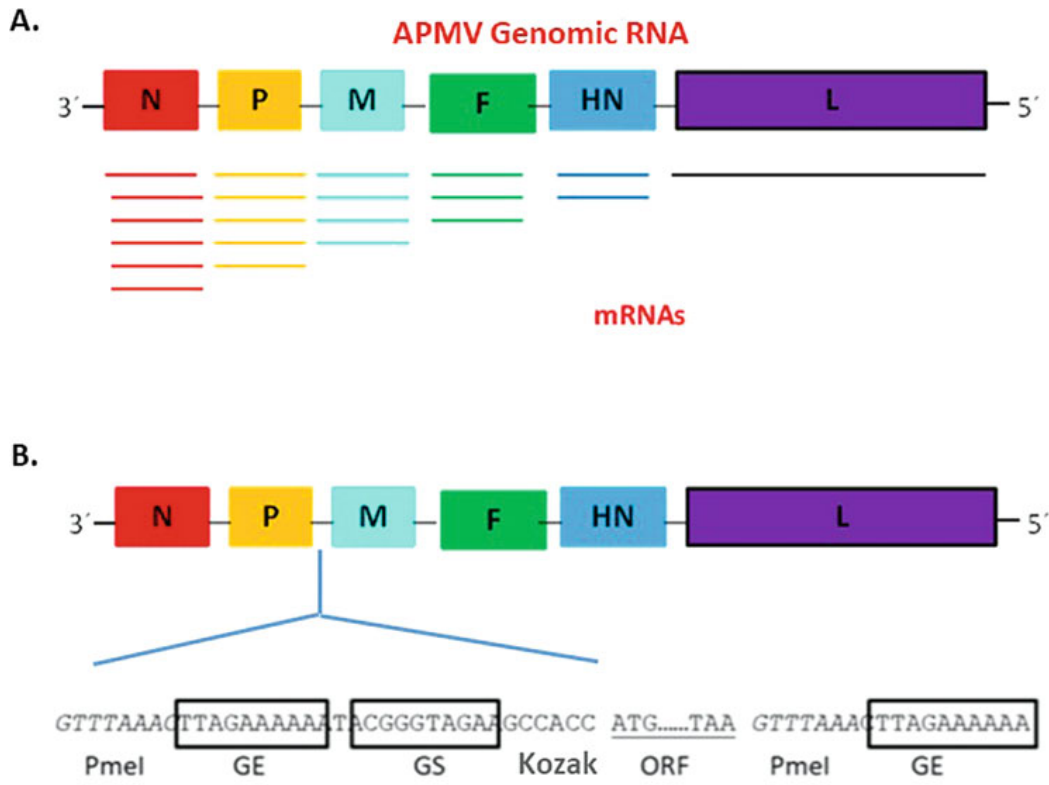
Fig. 2 Genomic organization and transcription scheme of APMVs (**a**). Construction of full-length genome of APMV vector containing the gene of protective antigens (**b**). The ORF of protective antigen is flanked by gene-start and gene-end signals of respective virus and inserted into the vector. This figure is illustrating the insertion of an antigen into intergenic region between the P and M genes in a full-length antigenomic cDNA of recombinant NDV

optimization of the location for the insertion of the gene cassette. Due to a polar gradient transcription, foreign genes are expressed more efficiently when placed closer to 3 end of the genome (Fig. 2a) [1]. In many cases, NDV and other APMV vectors have shown to efficiently express the foreign protein at the insertion site between the P and M genes (Fig. 2b) [5]. In case of APMV-3, one optimization study showed that the insertion site between the N and P genes was found optimal for efficient expression of the foreign protein [12]. This can be variable depending on the types of protective antigens, thus requiring for their optimization in vaccine construct design. NDV has also shown to accommodate a foreign gene (at least 4.5 kb in length) with a good degree of stability [2] and to express two different protective antigens simultaneously [16].

1. For the construct, the gene of protective antigen can be primarily prepared by PCR amplification (*see* **Note 9**). Amplify a foreign gene using the Platinum Taq DNA polymerase following the manufacturer's instruction. Mix with 50 ng of DNA as a template, primers, and dNTPs. Use the following cycling

parameters:1 cycle, 2 min 94 °C (initial denaturation); 25 cycles, 30 s 94 °C (denaturation), 30 s 56 °C (annealing), and 2 min 72 °C (extension); and 1 cycle, 5 min 72 °C (final extension).

2. Mix the PCR product with a loading dye. Load the sample and DNA ladder on a 1% agarose gel and run the gel with TAE electrophoresis buffer. Visualize the gene on a UV transilluminator, and cut the band using a razor blade. Place the gel slice in a 1.5-ml microcentrifuge tube and purify using a DNA purification kit following the manufacturer's instruction. Determine the DNA concentration using a spectrophotometer.

3. Digest purified gene (1–2 μg) with restriction enzyme for 3 h at 37 °C and subsequently purify using a PCR purification kit following the instructions.

4. Linearize full-length APMV plasmid (1–2 μg) with restriction enzyme for 5 h at 37 °C and consequently, dephosphorylate by directly adding alkaline phosphatase (1 μl) and incubating for 1 h at 37 °C to prevent self-ligation. Conduct purification using a DNA purification kit following the instructions. This step can also eliminate a heat inactivation procedure required for the enzymes.

5. Conduct the ligation reaction with ligase overnight at 16 °C according to manufacturer's instructions.

6. Next day, conduct the transformation by adding the ligation mixture (2–3 μl) into DH10B competent cells (50 μl) in a 1.5-ml microcentrifuge tube for 30 min on ice. Process heat shock for 45 s at 42 °C and cool it for 2 min on ice. Add 500 μl low-salt LB broth into the transformation mixture and incubate for 2 h at 30 °C with shaking.

7. Subsequently, take 100 μl and plate on a low-salt LB agar plate supplemented with tetracycline and incubate overnight at 30 °C (*see* **Note 10**).

8. For screening positive clones, pick individual colonies and inoculate into 3 ml of LB broth supplemented with tetracycline in 15-ml polypropylene tubes. Grow overnight in a 30 °C rotating incubator (*see* **Note 9**).

9. Isolate plasmids from the overnight cultures using a mini plasmid preparation kit following the manufacturer's protocol and confirm positive clones by conducting restriction digestion with enzyme. Positive clones will show two bands corresponding to the size of APMV vector (approximately 20 kb) and the gene of insert on the agarose gel.

10. Conduct sequencing analysis to confirm correct insert of the gene into APMV vector.

11. For the confirmed clone, prepare 200 ml of bacterial culture by overnight incubation at 30 °C. Process midi plasmid preparation followed by the manufacturer's manual. Determine the plasmid concentration for transfection experiment.

3.2 Recovery of APMV Vectored Vaccine

For the recovery of recombinant APMV from a plasmid based system, the T7 RNA polymerase can be provided by: (1) infecting the cells with recombinant virus expressing the T7 gene (i.e., vaccinia virus and fowl pox virus) and (2) using a cell line constitutively expressing the T7 polymerase (e.g., baby hamster kidney-21 cells, BSR T7/5) [17, 18]. This protocol is based on the infection of a modified vaccinia virus Ankara (MVA) in HEp-2 cells.

1. Prepare HEp-2 cells in DMEM supplemented with 10% FBS and penicillin/streptomycin in a 6-well plate (1×10^6 cells per well) day before transfection. In general, 80–90% confluent cells are suitable for the transfection.

2. Prepare the transfection mixture in a biosafety cabinet. First, prepare a mixture of plasmids (2 μg pTM1-N, 1 μg pTM1-P, 0.5 μg pTM1-L, and 5 μg pLaSota-HA per well) in Opti-MEM in a 1.5-ml microcentrifuge tube (making up to a total of 100 μl with Opti-MEM). Subsequently, dilute Lipofectamine 2000 (10 μl) into 90 μl Opti-MEM, incubate for 5 min at room temperature, and transfer into the plasmid mixture. Incubate the transfection mixture for 20 min at room temperature. The support plasmids can be constructed by individually cloning cDNA fragments containing the ORFs of the N, P, and L genes of APMV vector into an expression plasmid (e.g., pTM-1 and pGEM).

3. Prepare MVA-T7 in Opti-MEM at a multiplicity of infection of 1 pfu/cell (1 ml for each transfection experiment).

4. Wash the HEp-2 cells in the 6-well plate twice, each time with 2 ml PBS. Add 800 μl of prepared MVA-T7 into the transfection mixture, briefly mix by pipetting up and down, and infect the cells. Incubate the plate for 5 h at 37 °C, 5% CO_2 and replace the transfection medium with 2 ml DMEM supplemented with penicillin/streptomycin. To enhance the transfection efficiency, freshly collected allantoic fluid (10%) can be supplemented into the DMEM (optional). Incubate the plate for 3 days at 37 °C, 5% CO_2. During the incubation, HEp-2 cells typically show cytopathic effect (CPE) due to MVA-T7 infection.

5. For the propagation of recovered virus, collect the culture medium, inoculate 100 μl each into the allantoic cavity of 9- to 10-day-old SPF embryonated chicken eggs (2 eggs for each transfection) and incubate for 2 days 37 °C. If embryonated eggs are not available for the inoculation immediately, the

collected supernatant can be stored for 3–4 days at 4 °C. The supernatant can also be passaged in the chicken embryo fibroblast cell line (DF1). However, passaging in chicken eggs is more efficient for the propagation of recovered viruses than passaging in DF-1 cells.

6. Chill the infected eggs for 2–4 h or overnight at 4 °C. Place the eggs in a biosafety cabinet, spray the eggs with 70% ethanol, crack eggshells, and collect the allantoic fluid in a 15-ml centrifuge tube [19]. Centrifuge for 10 min at 600 × g, 4 °C, for clarification. Transfer the supernatant into a 15-ml tube.

7. Confirm the recovery of vaccine virus by hemagglutination (HA) assay using 1% chicken RBC. HA assays are carried out in a V-bottom 96-well plate. Pipette 50 μl of PBS per well in a V-bottom 96-well plate. Pipette 50 μl of the collected allantoic fluid into the wells in the first column of the plate. Make twofold serial dilutions. Include a negative control by pipetting 50 μl PBS in a row. Add 50 μl of 1% chicken RBC into each well. Incubate the plate for 20–30 min at room temperature or until a clear pellet is formed in the negative control wells.

8. After confirming the virus recovery, passage the virus into 9- to 10-day-old SPF embryonated chicken eggs. Plaque purify recovered virus as follows in the proceeding steps.

9. Prepare DF-1 cells in a 12-well plate (1×10^6 cells per well) the day before plaque purification experiment.

10. Make a 10-time serial dilution of allantoic fluid of recombinant virus in DMEM.

11. Aspirate the culture medium of DF-1 cells in the 12-well plate. Wash the cells two times, each time with PBS and inoculate 100 μl of diluted virus into the washed cells (10^{-3} to 10^{-8}) in duplicate. For virus adsorption, incubate the plate for 1 h at 37 °C, 5% CO_2. Gently, rock the plate 3 to 4 times during incubation.

12. Aspirate the inoculated diluents and wash the cells twice, each time with 1 ml PBS. Overlay the cells with methylcellulose medium (2 ml for each well) supplemented with 1% FBS and 10% allantoic fluid. Avirulent NDV strains (e.g., LaSota) and APMV-3 require an exogenous protease for efficient cleavage of the F protein for virus infectivity and replication. Freshly collected allantoic fluid can be used as an exogenous protease.

13. Incubate the plate for 5 to 6 days until plaques can be clearly visible.

14. Pick individual plaques using a sterile micropipette tip, dilute with 500 μl PBS in a 1.5-ml microcentrifuge tube, and vortex well.

15. Inoculate into SPF embryonated chicken eggs (100 μl each into 2 eggs) and incubate for 3 days.

16. Chill the eggs for 2–4 h at 4 °C and harvest allantoic fluid.

17. Confirm the presence of gene of protective antigen using a RT-PCR assay and its protein expression using western bot analysis. Determine the titer of virus, divide into 0.5-ml cryogenic tubes and store up to 1 year at −80 °C.

18. Stability of the gene insert in the NDV genome can be evaluated by in vivo passing in 1-day old chickens. After several passages, the recovered virus can be isolated and the presence of the gene insert can be confirmed by RT-PCR and DNA sequencing analysis.

4   Notes

1. For the amplification of foreign gene, forward and reverse primers can be designed by including the sequences of restriction enzyme site, Kozak and GS and GE of APMV vector (Fig. 2b).

2. dNTP mix (dATP, dCTP, dGTP, and dTTP; 10 mM) can be prepared by mixing 200 μl 100 mM dATP (10 mM final), 200 μl 100 mM dCTP (10 mM final), 200 μl 100 mM dGTP (10 mM final), 200 μl 100 mM dTTP (10 mM final), and 1200 μl sterile DNase/RNase free H$_2$O. Gently mix using a vortex mixer, divide into 100-μl aliquots each into a 1.5 ml of microcentrifuge tube and store at −20 °C following the manufacturer's expiration date.

3. For Agarose-TAE gel (1%), add 1 g agarose (Molecular Biology Grade) in 100 ml of 0.5× TAE, microwave until completely dissolved, and then add 2 μl ethidium bromide. Prepared gel can be stored at 60 °C in an oven.

4. For TAE buffer (0.5×), take 10 ml of 50× TAE buffer (242 g Tris base, 57.1 ml glacial acetic acid, and 100 ml of 0.5 M EDTA (pH 8.0); and adjust the final volume to 1 l with deionized H$_2$O) and adjust the final volume to 1 l with deionized H$_2$O. This prepared buffer can be stored up to 2 months at room temperature.

5. For low-salt LB culture medium, weigh 10 g Bacto-Tryptone, 5 g NaCl, and 5 g Yeast Extract, adjust the final volume to 1 l with deionized H$_2$O, and autoclave the medium. To make the low-salt LB plate, add 15 g agar before autoclaving, and store up to 2 months at 4 °C

6. Tetracycline (5 mg/ml; 1000×) stock can be prepared by adding 0.25 g tetracycline in 50 ml ethanol. Keep the solution

at −20 °C for 1 week to completely dissolve and store up to 6 months at −20 °C.

7. For preparation of phosphate-buffered saline (PBS), weigh 8 g NaCl (137 mM), 0.2 g KCl (2.7 mM), 1.44 g Na_2HPO_4 (10 mM), and 0.24 g KH_2PO_4 (1.8 mM); dissolve these reagents in 800 mL of H_2O; adjust the pH to 7.4 with HCl; and add H_2O to 1 l.

8. For methylcellulose medium, add 4 g methyl cellulose into an autoclaved bottle containing a stir bar, add 500 ml DMEM with 5 ml antibiotics (100× penicillin/streptomycin) and 5 ml fetal bovine serum (FBS), place on a stirring plate for about 1 week at 4 °C until completely dissolved. The prepared medium can be stored up to 6 months at 4 °C.

9. In case the size of the insert gene is >2 kb, the amplified gene can be inserted into a subcloning vector, digested with restriction enzyme, and then ligated into a linearized APMV backbone.

10. The size of APMV vector is approximately 20 kbp. The plasmid needs to be handled carefully for cloning and transfection experiments. The incubation temperature for the bacterial plate and culture for the cloning experiment is recommended at 30 °C to prevent any potential mutation of the plasmid.

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Part III

Vaccines for Poultry

<div style="text-align: right">**Chapter 4**</div>

Reverse Genetics and Its Usage in the Development of Vaccine Against Poultry Diseases

Barnali Nath, Sudhir Morla, and Sachin Kumar

Abstract

Vaccines are the most effective and economic way of combating poultry viruses. However, the use of traditional live-attenuated poultry vaccines has problems such as antigenic differences with the currently circulating strains of viruses and the risk of reversion to virulence. In veterinary medicine, reverse genetics is applied to solve these problems by developing genotype-matched vaccines, better attenuated and effective live vaccines, broad-spectrum vaccine vectors, bivalent vaccines, and genetically tagged recombinant vaccines that facilitate the serological differentiation of vaccinated animals from infected animals. In this chapter, we discuss reverse genetics as a tool for the development of recombinant vaccines against economically devastating poultry viruses.

Key words Reverse genetics, Poultry, Vaccines, Avian influenza, Newcastle disease, Avian coronavirus

1 Introduction

Reverse genetics is a method to study the *unknown function* of a known gene. This approach is opposite to the traditional forward genetics, where an *unknown gene* is studied for a known function. Reverse genetics was feasible after the introduction of recombinant DNA technology.

Reverse genetics: Known gene/protein—mutate the gene—explore resulting mutant phenotype.

Forward genetics: Known mutant phenotype—screen mutants—identify the gene(s) causing the phenotype.

1.1 Reverse Genetics in the Context of Virology

It starts with the engineering of mutations in known viral genes (DNA or cDNA) and the subsequent recovery of infectious viral particles to explore the unknown function of the viral genes or the resultant phenotype.

The first reverse genetics system for an RNA virus was established for the Poliovirus, a positive-sense RNA virus [1]. For a

negative-sense RNA virus, the minimum infectious unit is not an RNA molecule, but a core structure called ribonucleoprotein complex (RNP). In functional RNPs, the genomic RNAs have to be encapsulated with the nucleoprotein (N) and form a complex with the polymerase (L) and phosphoprotein (P). Due to technical difficulties in reconstituting biologically active RNPs, genetic manipulation of negative-sense RNA viruses has lagged than that of the positive-sense RNA viruses. In 1994, Schell, Mebatsion and Conzelmann first reported the recovery of Rabies virus, a non-segmented, negative-sense, RNA virus belonging to the family of *Rhabdoviridae*, entirely from cDNA. Reverse genetics of negative-sense RNA viruses progressed rapidly in the next years, as documented by the generation not only of non-segmented negative-sense RNA viruses [2, 3] but also of segmented negative-sense RNA viruses, including Bunyamwera virus [4] and Influenza viruses [5–7].

The use of vaccines is the most effective and inexpensive way of combating veterinary diseases. In veterinary medicine, reverse genetics is widely used to develop safe and effective live vaccines, broad-spectrum vaccine vectors, genetically tagged recombinant viruses—they facilitate the serological differentiation of vaccinated animals from infected animals (DIVA approach). Reverse genetics is also used to investigate the structure and function of viral genes and their proteins, study the interaction of viral proteins with host receptors and develop gene therapy tools [8].

1.2 Reverse Genetics Platform for the Development of Poultry Vaccines

Reverse genetics technology has been extensively applied for the development of vaccines against both DNA and RNA viruses of veterinary importance. This chapter focuses on the role of reverse genetics in developing poultry vaccines that are widely successful in multiple challenges and seroconversion studies under controlled conditions. We will discuss one example, each of segmented negative-sense RNA virus, non-segmented negative-sense RNA virus, and positive-sense RNA virus, which distresses the poultry industry globally.

1.2.1 Avian Influenza Virus

Avian influenza viruses are highly contagious and variable viruses that mainly affect birds. They are of two types: low pathogenic avian influenza (LPAI) viruses and highly pathogenic avian influenza (HPAI) viruses. LPAI viruses, while circulating in poultry flocks, are capable of evolving into HPAI viruses. HPAI viruses can devastate the poultry industry due to the high rate of mortality and morbidity associated with it. HPAI viruses can cause epidemics that spread rapidly and result in severe trade restrictions [9, 10]. Avian influenza viruses can transmit to mammals, including humans, after close/prolonged contact with infected poultry

[10]. Avian influenza viruses commonly reported from human clinical cases are the Asian lineage H5N1 HPAI viruses and the recent H7N9 LPAI viruses in China [11–14].

Avian influenza is caused by Influenza A virus (IAV) belonging to the *Orthomyxoviridae* family of RNA viruses. Influenza A virus consists of 8 negative-sense, single-stranded viral RNA gene segments that encode 11 functional proteins: polymerase basic (PB) 2, PB1, PB1-F2, polymerase acidic (PA), nucleoprotein (NP), hemagglutinin (HA), neuraminidase (NA), matrix (M) 1, M2, non-structural protein (NS) 1, and nuclear export protein (NEP; previously known as NS2) [15]. HA and NA are the major immunogenic surface glycoproteins that help in the entry and exit of the virus. HA is responsible for attachment to host cells via sialic acid (SA) receptors, and NA cleaves cell-surface SA to release the newly packaged virus from host cells. The classification of IAVs into subtypes is based on the genetic and antigenic properties of the surface proteins HA and NA. To date, 16 HA (H1–16) and 9 NA subtypes (N1–9) of IAVs are isolated from aquatic birds—the natural host of IAV [16].

In the case of IAV, reverse genetics has been extensively exploited to produce inactivated and live influenza vaccines, develop universal influenza vaccines, develop influenza virus-based vaccine vectors, dissect the roles of influenza virus gene segments in disease pathogenicity, and understand host–pathogen interactions [17].

Inactivated and Live Influenza Vaccines

Antigenic differences between the circulating and vaccine strains of a virus result in vaccine failure. Vaccine failure leads to the death of vaccinated poultry or their survival with a shedding virus. Consequently, this leads to an endemic situation. Since IAVs can also cause human infection due to reassortment and transmit from human to human, there is a high chance of global pandemic. This problem of antigenic differences between the circulating and vaccine strains can be resolved by producing recombinant viruses using the reverse genetics method. Targeted mutation in the HA protein is generally used as a tool to develop live/inactivated vaccines against influenza (Table 1).

Promising vaccine candidates against highly pathogenic avian influenza viruses (HPAIVs) of the H5N1 subtype was developed by Uchida and his team using reverse genetics [18]. The vaccine candidate strains contained: HA gene from the H5N1 subtype HPAIV, attenuated by mutation at the cleavage site; NA gene from the H5N1 subtype, or the H5N3 subtype; and internal genes from A/Puerto Rico/8/34 strain of IAV. They generated an inactivated recombinant vaccine strain. When this vaccine was administered with oil-emulsion, it completely protected chickens from a homologous viral challenge. The higher dose of antigen was

Table 1
Targets and techniques for attenuation of poultry viruses using reverse genetics

Virus	Target for attenuation	Modification	Reference
Avian influenza virus	Hemagglutinin gene (HA)	Mutation/removal of the multi-basic amino acid motif "RERRRKKR↓GLF"	[18–21]
	Non-structural protein 1 gene (NS1)	Truncation of the C-terminal of NS1 protein	[22]
	Receptor binding domain (RBD)	Amino acid substitutions (K193E and G225E) in the RBD	[23]
Newcastle disease virus	Fusion gene (F)	Virulent F protein cleavage site motif "RRQKRF" mutated to avirulent motif "GRQGRL" by three amino acid substitutions	[24–26]
	Hemagglutinin-neuraminidase gene [HN]	Deletion of the 5′ UTR of the HN gene	[27]
	Non-structural protein gene V and W	Deletion of the NDV genes V and W that act as interferon antagonists	[28]
Infectious bronchitis virus	Spike protein gene (S)	Mutation of S protein gene	[29]
	Accessory genes 3 and 5a	Deletion of 3 and 5a by targeted RNA recombination	[29, 30]
	Replicase gene encoded proteins	Amino acid substitutions in replicase gene encoded proteins, e.g., V342D, S1493P, P2025S, F2308Y	[31]

also effective in increasing survival and reduction of viral shedding even when challenged by an H5N1 virus of a different clade. The vaccine candidate also facilitated the differentiation of infected from vaccinated animals (DIVA). It was demonstrated against a challenge with H5N1 HPAIVs when the recombinant H5N3 subtype viruses were used as the antigens for the vaccine [18].

A reverse genetics-based rgH5N2 inactivated vaccine that protects against a high dose challenge of the H5N1 avian influenza virus in chicken was generated by plasmid-based reverse genetics system with WSN/33/H1N1 as backbone virus [19]. The vaccine candidate strains contained: H5-HA gene from H5N1 virus (A/chicken/West Bengal/80995/2008) of antigenic clade 2.2, attenuated by mutation of the basic amino acid cleavage site RRRKKR*GLF to IETR*GLF; N2-NA gene from H9N2 field isolate (A/chicken/Uttar Pradesh/2543/2004) [19].

Similarly, a broadly reactive influenza vaccine was developed to cope with the continuous antigenic evolution of influenza viruses by mutating the HA protein [20].

1.2.2 Newcastle Disease Virus

Newcastle Disease (ND) is one of the notable viral diseases of the poultry industry. It causes substantial economic losses due to a high rate of mortality, commercial restrictions and control measures, especially in developing countries.

ND is caused by the Newcastle disease virus (NDV), the prototype *Avulavirus* in the family *Paramyxoviridae* [32, 33]. NDV consists of a non-segmented, negative-sense, single-stranded RNA genome that encodes six essential proteins, viz. nucleoprotein [N], phosphoprotein [P], matrix [M], fusion [F], hemagglutinin-neuraminidase [HN], and RNA-dependent RNA polymerase [L] [32–34]. The surface glycoprotein F is the major protective antigen of NDV [34]. Based on disease signs and lesions, NDV has been classified into lentogenic (low virulent), mesogenic (moderately virulent), and velogenic (highly virulent) [34, 35].

Reverse genetics has been used to develop: better attenuated and a genotype-matched vaccine against NDV, broad-spectrum NDV vaccine vector, and bivalent NDV-vectored vaccines against poultry viruses.

A better Attenuated and Genotype-Matched Vaccine Against NDV

The current NDV vaccines Hitchner B1 and LaSota are naturally occurring strains that were developed into live-attenuated vaccines. So, there is a risk of these vaccines to cause disease due to some unfortunate reversion to virulence. Also, the current vaccines, isolated around 65 years ago, belong to genotype II of class II of NDV. However, NDV, being an RNA virus, is a continually evolving virus. The circulating strains associated with NDV outbreaks worldwide predominantly are from genotypes V, VI, VII, and XIII of class II [36–41]. The genotypically distant current vaccines can only offer a decent protection against the virus and allow significant breakthrough infection and virus shedding. In such a situation, the virus recirculates in the environment and acquires adaptive changes in response to immune pressure [42].

Reverse genetics can be used for the development of better NDV vaccines by introducing mutations in the F and HN genes. The NDV F cleavage site is the major molecular determinant of NDV virulence. Better NDV vaccines can also be developed by deleting the V and W genes of NDV that act as interferon antagonists. This deletion will make the vaccines more attenuated, but still immunogenic. This kind of vaccines can also be used for *in ovo* vaccination [28]. Reverse genetics is also used to develop genotype-matched vaccines for NDV, which reduces viral shedding and provides better protection [24] (Table 1).

Bivalent NDV-Vectored Vaccines Against Poultry Viruses

NDV consists of a modular genome with only six essential genes, shows the least recombination with the host genome, and elicits both humoral and cellular immune response [34]. These features have attracted many scientists to develop NDV into a broad-spectrum vaccine vector against several animal and human

pathogens. In the case of poultry, bivalent NDV-vectored vaccines are developed using reverse genetics [43]. NDV vaccines can be combined with other poultry vaccines and can be used as a bivalent vaccine to control economically important poultry diseases [44]. The immunogenic foreign protein of other poultry viruses can be inserted in the NDV vaccine vector or backbone. Recombinant NDV expressing the foreign protein shows a high and stable expression of foreign protein after many passages, both *in vitro* and *in vivo* [43]. Moreover, the production of recombinant NDV-vectored bivalent vaccines is highly cost-effective since they grow to very high titers in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs and cell culture.

Bivalent NDV-vectored poultry vaccines have been developed by expressing HA genes of HPAIV (A/H5) [45, 46] and (A/H7) [47]; VP2 gene of infectious bursal disease virus (IBDV) [48]; S2 gene of avian coronavirus infectious bronchitis virus (IBV) [49]; gB, gD, gC genes of infectious laryngotracheitis virus (ILTV) [50, 51]; G gene of avian metapneumovirus (aMPV) in recombinant NDV backbone [44, 52].

DIVA Strategy

Reverse genetics can be used to generate a recombinant chimeric NDV vaccine that allows serological differentiation between vaccinated and infected animals. A marker virus was designed for NDV, in which HN from avian paramyxovirus type 4 replaced the HN gene of NDV. Hence, it facilitated the differentiation between vaccinated and naturally infected animals based on different antibody profiles against HN proteins [53].

1.2.3 Avian Coronavirus (Infectious Bronchitis Virus)

Infectious bronchitis (IB) is another contagious disease of the poultry with grave economic implications. The disease causes retardation in the sexual maturity of birds by damaging their reproductive organs beyond repair, leading to reduced fertility, hatchability, egg quality. The consequences are "false layers syndrome" and high mortality [54, 55].

IB is caused by the infectious bronchitis virus (IBV), which belongs to the genus *Gammacoronavirus* within the family *Coronaviridae* [54, 56]. IBV consists of one of the longest, positive-sense, single-stranded RNA genome. It encodes for both structural (spike protein [S], envelope protein [E], membrane glycoprotein [M] and nucleoprotein [N]) and non-structural (product of gene 1, 3 and 5) proteins [57–59]. The S protein, located on the surface of the viral membrane, is involved in viral attachment with the host cell receptor and fusion of the virion with the cell membrane [60–62]. It is post-translationally cleaved at a multi-basic cleavage site into the amino-terminal S1 and the carboxyl-terminal S2 subunits [63–65].

Several scientists are applying reverse genetics technology to increase the stability and efficacy of the traditional IBV vaccines by modifying one or more viral genes [66–68]. For example, two

separate groups, Casias et al. and Armesto et al., constructed recombinant BeauR-IBV vaccines by substituting the antigenic S1-glycoprotein of avirulent Beau-IBV strain with S1-gene from pathogenic M41 and European 4/91 strains, respectively [69, 70]. A recombinant H120 (R-H120), was constructed by Zhou and his team, which conferred a protection rate comparable to intact H120-vaccine [71]. Recombinant live-attenuated IBV vaccine candidates have also been developed by targeted RNA recombination [30, 72] (Table 1).

2 Materials

2.1 Cell and Viruses

1. 1× Phosphate-buffered saline (PBS).
2. Dulbecco's Modified Eagle Medium (DMEM).
3. Fetal Bovine Serum (FBS).
4. Antibiotic-Antimycotic (100×) solution.
5. 0.25% Trypsin-EDTA.
6. 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs.
7. Sorvall™ WX+ Ultracentrifuge.

2.2 Reverse Genetics Construction

1. TRIzol™ Reagent (Invitrogen).
2. High-Capacity cDNA RT Kit (Applied Biosystems).
3. Primers for the complete genome sequence of the virus.
4. High-Fidelity DNA polymerases.
5. Q5$^®$ Site-Directed Mutagenesis Kit (NEB).
6. Restriction enzymes (NEB).
7. Cloning and expression plasmids.
8. T4 DNA Ligase (NEB).
9. High transformation efficient DH10B Competent Cells (NEB).

2.3 Transfection and Recovery

1. Opti-MEM.
2. Modified vaccinia virus strain Ankara expressing the T7 RNA polymerase (MVA/T7).
3. Lipofectamine™ 2000 Transfection Reagent (Invitrogen).
4. mMESSAGE mMACHINE™ T7 Transcription Kit (Invitrogen).
5. Chicken red blood cells (RBC).
6. Trypsin, TPCK-Treated.
7. Trypsin acetylated from bovine pancreas.
8. Gene Pulser Xcell™ Total System (BIO-RAD).

2.4 Characterization of the Recombinant Virus

1. Methylcellulose.
2. 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs.
3. 1-day-old SPF chicks.

2.5 Immunization and Challenge

1. 4-week-old SPF chicken.
2. Plain DMEM.
3. 1× PBS.
4. Antibiotic-Antimycotic (100×) solution.
5. Cotton swabs/Applicators.
6. Microcentrifuge tubes/Falcon tubes.
7. Vacutainer (untreated), syringes, tubes for blood collection and serum preparation.

3   Methods

3.1 Cells and Viruses

1. Maintain Vero (African green monkey kidney), 293 T (Human embryonic kidney 293 T), MDCK (Madin-Darby canine kidney), Hep-2 (Human epithelial type 2), DF-1 (Chicken embryo fibroblasts) and BHK-21 (Baby hamster kidney) cells in DMEM supplemented with 10% fetal bovine serum (FBS) and 1× antibiotic and antimycotic solution at 37 ° C and 5% CO_2 (*see* **Note 1**).
2. Propagate the viruses: HPAIV (H5N1) strain, PR8 (H1N1) [A/Puerto Rico/8/1934] strain, NDV velogenic strain, IBV vaccine strain H120 (live-attenuated vaccine strain of Massachusetts serotype) and pathogenic strain in the allantoic cavity of 10-day-old SPF embryonated chicken eggs. Harvest the infected allantoic fluids 48–96 h post-inoculation depending on its virulence. Purify the viruses partially by ultra-centrifugation in a discontinuous sucrose gradient (30% and 55% sucrose).

3.2 Reverse Genetics Construction and Sequencing

3.2.1 Avian Influenza

Isolate the genomic RNA from the PR8 strain (H1N1) using TRIzol Reagent and subject it to reverse transcriptase-polymerase chain reaction (RT-PCR) to amplify and clone all eight gene segments using cDNA synthesis kits and high-fidelity DNA polymerases. For transcription, full-length genes NS, M, NP, PA, PB1, and PB2 of PR8 strain are cloned into pPollSapIT plasmid as a vector. For protein expression, full-length open reading frames (ORFs) of PB1, PB2, PA, and NP of PR8 strain are cloned into pDNA3.1 mammalian expression vector (Invitrogen). To antigenically match the vaccine to HPAIV (H5N1), amplify the full-length NA and HA genes of HPAIV by RT-PCR and clone them into the pPolISapIRib (pPSR) vector. A total of 12 plasmids are constructed
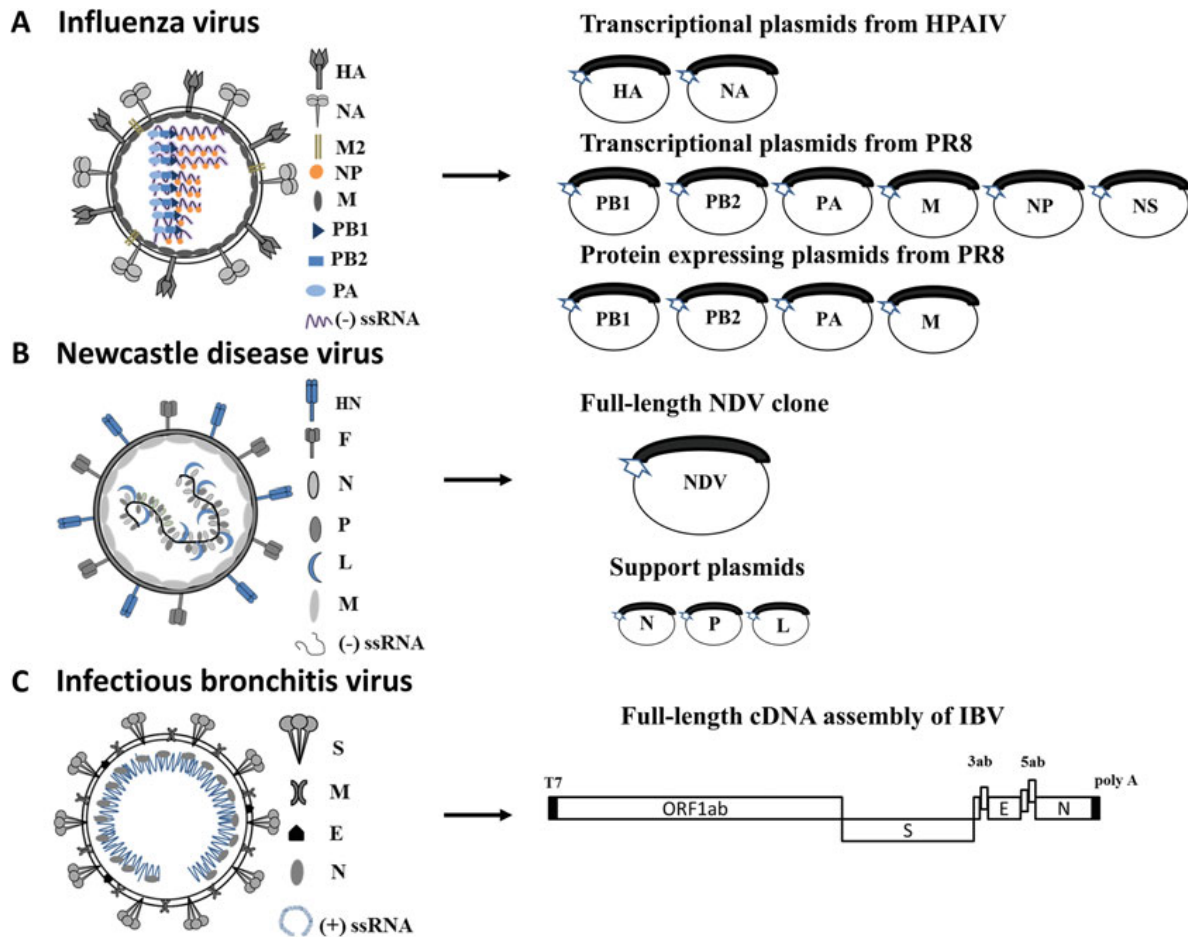
Fig. 1 Overview of reverse genetics approach for developing a vaccine in poultry: (**a**) Influenza virus plasmid-based reverse genetics: the HA with mutated cleavage site and NA from HPAIV (H5N1), the other ten plasmids are from non-pathogenic PR8 strain. (**b**) Newcastle disease virus plasmid-based reverse genetics: full-length cDNA clone of NDV and three support plasmids. (**c**) Infectious bronchitis virus reverse genetics: An *in vitro* assembled full-length genomic cDNA of IBV

[5]. For the development of live and inactivated vaccine strains, modify the virulent associated multi-basic amino acid cleavage site of the HA gene of HPAIV (H5N1) strain to that of LPAIV (RRKKR↓GLF to RETRF↓GLF) by site-directed mutagenesis [21] (Fig. 1a).

3.2.2 Newcastle Disease Virus

Clone the complete cDNA antigenome of a velogenic strain of NDV into a high-copy number cloning vector - pUC19 vector [73]. It can be achieved by cloning the viral genome in fragments that are generated by RT-PCR of viral RNA isolated from NDV infected allantoic fluid. The cloning should be accurate; addition or deletion of nucleotides from the viral genome will lead to the disruption of the "rule of six"—critical for the packaging of NDV virions [74]. Sequentially clone the NDV fragments into the pUC19 vector between the T7 promoter and the hepatitis delta

virus ribozyme (HDR) sequence and the T7 terminator sequence. To attenuate the velogenic strain of NDV, convert the virulent F gene cleavage site into an avirulent cleavage site ("RRQKR↓F" to "GRQGR↓L") by site-directed mutagenesis and replace it into the full-length cDNA clone [25]. Three support plasmids of N, P and L genes are needed to recover the virus as they form the ribonucleo-protein complex (RNP), which is essential for converting the viral genomic RNA into individual proteins and even synthesis of geno-mic RNA for progeny virus. So, it is necessary to clone the ORFs of N, P, and L into a mammalian expression vector, e.g., pcDNA3.1 (Invitrogen) (Fig. 1b).

3.2.3 Infectious Bronchitis Virus

Amplify the complete genome of the H120 strain of IBV into ten PCR fragments by RT-PCR and clone them using *BsmB*I or *Bsa*I restriction enzymes, either at the 5′ or 3′ ends of a pMD19-T vector [71, 75, 76]. Digest all the fragments with the respective restriction enzymes and purify them by agarose gel purification. Assemble the complete cDNA of IBV by orderly ligation and then use the whole construct as a template for *in vitro* translation [71]. Incorporate the T7 promoter at the 5′ end of the first fragment and poly-A tail at the 3′ end. Separately clone the N gene into a pMD19-T vector with a T7 promotor at 5′ end. The S1 fragment of the H120 vaccine strain can be replaced with S1 of the circulating pathogenic IBV strains by overlapping PCR (Fig. 1c).

3.3 Transfection and Recovery of the Recombinant Virus

3.3.1 Avian Influenza

Seed 293 T cells at 90% confluence in 6-well plates. To generate HPAIV/PR8 reassorted virus, two plasmids from HPAIV (HA and NA) and other ten from PR8 [six transcription plasmids (PB1, PB2, PA, M, NP and NS) and four protein-expressing plasmids (PB1, PB2, PA, and NP)] are used for transfection. Mix 1 μg of each plasmid with 12 μl of Lipofectamine 2000 reagent in 250 μl of Opti-MEM reagent for 20–30 mins at room temperature to form the DNA–lipid complex. Overlay this complex on 293 T cells and incubate at 37 °C for 4–6 h. Follow by replacement of DNA–lipid complex with fresh media. After 16–24 h, replace the media by 2 ml Opti-MEM containing 0.5 μg/ml TPCK-treated trypsin and incu-bate for an additional 48 h at 37 °C. Inoculate the cell-lysate into 10-day-old SPF embryonated chicken eggs and incubate for 48–72 h at 35 °C. After incubation, harvest the allantoic fluid and check for the presence of the virus with hemagglutination assay (HA) using 0.5% chicken RBC. Confirm the presence of the virus by HA and then re-passage the virus in eggs. Confirm for possible mutations by DNA sequencing.

3.3.2 Newcastle Disease Virus

Seed Hep-2 cells at 90% confluence in 6 well plates and infect the cells with 3 MOI of MVA-T7 for 1 h at 37 °C. After that, transfect the full-length cDNA clone and support plasmids in a ratio of 3:1.5:1:0.5 [NDV full-length clone (3 μg), N (1.5 μg), P (1 μg),

and L (.5 μg)]. Mix the plasmids with Lipofectamine 2000 reagent in Opti-MEM and incubate them at room temperature for 30 min. After incubation, discard the MVA-T7 infection mixture and wash the cells once with 1× PBS. Follow this by the addition of 1 ml fresh Opti-MEM to the wells. Add the transfection mixture drop-wise to the cells and incubate them for 4–6 h at 37 °C. Post-incubation, replace the transfection mixture with fresh DMEM containing 1 μg/ml of acetylated trypsin or 10% fresh allantoic fluid. 72 h post-transfection, collect the cells with media and freeze-thaw them three times. Centrifuge to clear the supernatant and inoculate into 10-day-old SPF embryonated chicken eggs. Post 5–7 days incubation, harvest the allantoic fluid and check for the presence of virus by HA. Re-inoculate the allantoic fluid with the virus into 10-day-old SPF embryonated chicken eggs to further amplify and characterize the recovered virus [77].

3.3.3 Infectious Bronchitis Virus

The complete genomic cDNA template of the H120 strain is used as a template for the synthesis of genomic RNA *in vitro* by mMES-SAGE mMACHINE® T7 kit. Similarly, the N gene transcript is also generated from the pMD19-N clone, which is required to enhance the recovery of IBV [78, 79]. Transfect both of the transcripts into BHK-21 cells by electroporation using the Gene Pulser Xcell™ Electroporation System (Bio-Rad). Post-transfection, seed the cells in DMEM supplemented with 10% FBS and incubate for 48 h followed by inoculation of the cell-lysate into 10-day-old SPF embryonated chicken eggs [80]. Confirm the recovery of the virus in the allantoic fluid by RT-PCR and check for modifications in the S1 gene by DNA sequencing.

3.4 Characterization of the Recombinant Virus

1. Passage the recombinant viruses ten times or more in 10-day-old SPF embryonated chicken eggs as well as in selected cell lines to check the stability of the foreign gene expression.

2. Perform a plaque assay to identify the vaccine strain of the virus. Seed the cell line of choice, and after the formation of a monolayer, infect the cells with 0.01 MOI of the recovered attenuated virus. After 1 h incubation at 37 °C, overlay the cells with 0.8% methylcellulose in DMEM with and without TPCK-treated trypsin. The recovered attenuated viruses fail to form plaques in the absence of trypsin [25].

3. Mean Death Time (MDT) for NDV: Make tenfold serial dilutions of fresh allantoic fluid containing the recovered virus in sterile saline (10^{-6} to 10^{-9} dilutions). Inoculate 100 μl of each dilution into ten 10-day-old SPF embryonated chicken eggs and incubate at 37 °C for 7 days. Monitor the eggs for any death of embryo every 12 h. The highest dilution of the virus where all the embryos are dead is known as the minimum lethal dose (MLT). The mean time in hrs for MLT is known as MDT.

Velogenic strains of NDV show <60 h MDT value, the vaccine generated by mutation of the F cleavage site, will show an MDT value of approximately 120 h.

4. Intracerebral pathogenicity index (ICPI) for NDV: Make similar dilutions as in MDT protocol and inoculate 1-day-old SPF chicks. Observe the birds for 8 days and score as 0 for normal, 1 for sick, and 2 for dead birds. Velogenic NDV strains show an index value near 2, whereas vaccine strains generated by mutation of the F cleavage site will show an index value near 0 [81].

5. Embryo dwarf test for IBV: Inoculate groups of 10-day-old SPF embryonated chicken eggs with IBV vaccine strain, pathogenic strain, vaccine strain generated with reverse genetics and mock control. The embryos of the pathogenic group show stunting and dwarfing, comparatively less or no effect will be seen in vaccine treated groups [75].

3.5 Immunization and Challenge Study

1. Divide 4-week-old SPF chickens into four groups: (a) unvaccinated unchallenged mock group, (b) challenge group, (c) vaccine control group, and (d) reverse genetics vaccine group.

2. Inoculate or inject the birds based on the vaccine. Two weeks post-vaccinations, challenge birds with the respective challenge strains except for the mock group. In case of HPAIV the vaccine is inactivated before inoculating birds (*see* **Note 2**).

3. Post-challenge, observe birds for signs, symptoms, and motility (*see* **Note 3**).

4. Collect tracheal and cloacal swabs on 3, 5, and 7 days post-challenge for viral titration to detect the shedding of the virus.

5. Collect serum samples from birds on day 0, before vaccination, 1 day post-challenge, and 14 days post-challenge from surviving birds for viral neutralization and Hemagglutination Inhibition (HI) against the challenge strain.

4 Notes

1. 293 T is the cell line of choice for IAV recovery because of high transfection efficiency and a higher rate of success in the recovery of the virus. MDCK is the most widely used cell line for IAV growth and propagation. Hep-2 cell line is used in NDV recovery as they resist the cytopathic effect from MVA infection. DF-1 and BHK-21 cell lines are used for the propagation of various poultry viruses.

2. The H5N1/PR8 vaccine virus is inactivated by treating the purified virus with 0.025% formalin at 4 °C for 3–4 days. The inactivation is checked by titration in embryonated eggs.

As the Influenza virus has a segmented genome, there is always a chance of reassortment of the vaccine strain converting it into virulent strains [82].

3. All the viruses are respiratory in nature; some common clinical signs include necropsy lesions in the upper respiratory tract, hemorrhages in trachea and lungs.

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Part IV

Vaccines for Farm Animals

Chapter 5

Use of a Ferret Model to Test Efficacy and Immunogenicity of Live Attenuated *Mycobacterium avium* Subspecies *paratuberculosis* Vaccines

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Abstract

Native hosts for the bacterial agent that causes Johne's disease are ruminants, which include cattle, sheep and goats among others. These large animals are often too costly to be used in testing experimental vaccines. In this chapter, we provide detailed methods to use an inexpensive and more manageable animal host, the ferret, to test efficacy and immunogenicity of live-attenuated *Mycobacterium avium* subspecies *paratuberculosis* (MAP) mutant strains prior to consideration as vaccine candidates.

Key words Mycobacterium, Animal model, Ferrets, Johne's disease, Paratuberculosis

1 Introduction

Animal models for paratuberculosis have been developed in rodents and ruminants [1, 2]; however, no studies were conducted on ferrets. Many studies with existing models used oral inoculation to simulate bacterial uptake via the fecal-oral route, which is the natural mode of infection. In one notable study, the infectivity via oral inoculation of calves with either a low passage MAP strain or MAP isolated from ileal mucosal scrapings was compared [3]. Tissue culture results showed that when calves ingest in vitro cultured MAP or MAP obtained from mucosal scrapings, MAP colonization was observed in multiple tissues. Ranking of tissue sites by the number of MAP positive cultures demonstrated preferential colonization of the jejunum, followed by the ileum, duodenum, and spiral colon and associated lymph nodes. Oral inoculation of calves, by any of the methods, was more effective, resulting in the greatest tissue involvement. In contrast, fecal shedding was minimal during

the study, regardless of inoculation method, and was detected sporadically. Multifocal granulomas were observed in the lymph nodes of the jejunum, ileum, ileocecal valve, and colon of calves in each treatment group, which is considered a hallmark characteristic of subclinical infection.

Although oral inoculation with mucosal scrapings from cows with clinical signs performed best in the neonatal calf model by Stabel and coworkers [3], this route is not appropriate to compare the virulence of MAP strains that could eventually be used as oral or intradermal vaccines. In this case, we observed a greater number of tissues with lesions when inoculated orally with *in vitro* cultured wild-type K-10 compared to an infection with mucosal scrapings. Moreover, it was determined that early immune markers of MAP infection were adequate for calves infected with strain K-10 [4]. Observations with K-10-infected calves showed a robust IFN-γ response upon stimulation of PBMCs with a MAP protoplasmic extract starting at 6 months post-infection that continued up to the 12-month duration of the study. Significant levels of iNOS secretion at 12 months post-infection were also observed along with strong lymphocyte proliferation responses. Finally, increases in CD4, CD8, and γδTCR T-cells positive for the activation/differentiation markers CD25, CD26, CD45RO, and CD5 were noted at 12 months post-infection [3]. Thus, we applied the oral route of administration to ferrets in setting up this animal model. Indeed, this route of delivery has shown significant mucosal colonization and the expected immunological response in a period of 8–12 months in calves [3, 4], and a shorter timeframe may occur in ferrets.

Ferrets have been used as a model for influenza virus [5, 6] and *Mycobacterium bovis* [7] among other bacterial and viral pathogens [8]. The respiratory anatomy of the ferret has similar characteristics to humans, making it ideal for respiratory pathogens. One potential advantage of using the ferret as a model for MAP infection is the short small intestine common to these animals, which makes the site of infection very focused but may also prevent efficient MAP adherence and infection to the intestine. Other advantages include the labor needed to care for these animals is considerably less than that required for cattle and a small animal model allows for housing more than can be done with calves. In addition, ferrets are naturally infected and serve as wildlife reservoirs for *M. bovis* and MAP [7, 9, 10]. Thus, ferrets provide a valid small animal host to test the virulence and transmission of these mycobacterial species as well as test the virulence of mutant strains intended for use in wildlife and domesticated ruminant species. Finally, they have a longer life span relative to mice, which are often used as another small animal model for mycobacteria. This makes ferrets ideally more suitable to studying chronic diseases.

The protocols listed herein were developed to test the safety and immunogenicity of two independently generated MAP mutants in the ferret model, but can also be applied to any live attenuated MAP vaccine-challenge study.

2 Materials

1. Live MAP knockout mutants along with the parent strain K-10 (wild-type).

2. Ferrets, female, de-scented (3–4 months old).

3. Cages
   (a) Dimensions:
   - Rack: 67″ W × 33″ D × 70″ H (external).
   - Cage: 27 1/8″ W × 27 1/8″ D × 18″ H (internal—5.1 sq. ft. floor area).

   (b) Three ferrets per cage.

4. Purified protein derivative (PPD) obtained from *M. bovis* strain AN-5 (lot #1909), or *M. avium* strain D-4 (serial number 30-EXP-1901) and MAP field strain (serial #134-1901). Source: National Veterinary Services Laboratories, Ames, Iowa, USA.

5. Critical care carnivore diet, powder (Oxbow Animal Health). Ferret High Density Diet 5L14.

6. Middlebrook 7H9 broth and 7H11 agar media (Remel™ media).

7. Mycobactin J (Allied Monitor).

8. MAP sonicated extract, prepared in-house.

9. Phorbol 12 myristate 13 acetate (PMA; TOCRIS).

10. Ionomycin.

11. Tween® 80.

12. ELISA kit (IDEXX).

13. Ferret IFN-γ ELISA assay. (MabTech)

14. Herrold's Egg Yolk Agar Slants with Mycobactin J and Amphotericin B, Nalidixic Acid, Vancomycin (HEYM tubes with ANV, Becton Dickinson-BBL™).

15. BD Vacutainer® plastic heparin collection tubes, 4 mL.

16. 96 round bottom well plates.

17. Microchips/transponders (IPTT-300 from Bio-Medic Data Systems).

3   Methods

3.1   Overall Experimental Timeline

The timeline below shows the overall infection and euthanasia schedule. This timeline does not show sample collection, which is further detailed in Subheading 3.2 below.

Prior to infection:

Week-2—ferrets arrive and are acclimatized in cages.

Week-1—Collect pre-infection samples—blood, feces, and record temperature.

Week-1 to 0—Check temperature for baseline and feed animals oxbow through a syringe once per day.

Post-infection:

Day 0—Infect with MAP wild-type and mutants in Oxbow feed. Measure initial animal weights.

Week 20—Euthanize ferrets and collect tissues.

3.2   Monitoring and Data Collection

Daily: Check overall health and activity of animals.

Weekly: Record weight, body temperature, and collect feces from the cage for culture and PCR.

Bi-weekly:

1. Collect blood for serum prep at time points –1, 1, 3, 5, 7 weeks post-infection (p.i.), and at termination.

2. Collect blood for PBMC prep at time points –1, 1, 3, 5, and 7 weeks p.i. Stimulate with MAP lysate and *M. avium* PPD. Collect supernatants for IFN-γ ELISA.

Monthly: Skin test wk 0, 4. and 8 p.i. with saline, MAP lysate, and Johnin PPD.

Termination: At 40 weeks post-infection, animals were euthanized and tissues were collected. Tissues included liver and mesenteric lymph nodes. The spleen was also collected to prepare splenocytes (stimulated with culture medium, MAP extract, PPD) phytohemagglutinin, and feces for CFU. Collect intestines for histopathology. Also, at the end point, blood is collected for serum preparation and PBMC stimulation with PPD, lungs, intestines for CFU and histopathology, feces for CFU and PCR.

3.3   Animals and Procedures

3.3.1   Animal Type and Handling

1. Twelve female ferrets, 3–4 months old, are allowed to acclimate for 10 days in BSL2 animal facilities (*see* **Note 1**).

2. During acclimation, the ferrets were also trained to eat their feed slurry through a 3-mL syringe (*see* **Note 2**).

3. Ferrets are fed with Critical Care Carnivore diet (Oxbow) once daily until the day of infection (*see* **Note 3**). After infection, they are fed dry pellets from LabDiet.

4. Each ferret is injected with a transponder subcutaneously into anesthetized animals during the collection of pre-infection samples. Using the transponder accompanying software, each ferret is readily identified by group, treatment and ferret number. Thus, animals equipped with these transponders are easily scanned for identity data.

5. Ferrets are divided in groups of 3 for vaccine efficacy testing.
   (a) Sham-inoculated control group
   (b) Wild-type K-10 infected group
   (c) Vaccinated and K-10 infected group

3.3.2 Blood Collection

1. Blood is collected by cranial vena cava puncture as described previously [11].

2. Collection is accomplished with a 25-gauge needle attached to a 3-mL syringe and can expect a 1-mL draw per animal.

3. Whole blood was either immediately processed for the IFN-γ assay (Subheading 3.4.3) or red cells were harvested and removed for serum used in ELISA assays (Subheading 3.4.4).

3.4 Infection of Ferrets

3.4.1 Culture and Inoculum Preparation

1. Two days before infection, pre-immune samples that include blood, nasal wash, throat swab and feces were collected and stored at $-80\,^{\circ}$C.

2. Each of the mycobacterial mutants and the wild-type strain are cultured in Middlebrook 7H9 supplemented with OADC and 2 mg of Mycobactin J per liter.

3. Cultures are incubated at $37\,^{\circ}$C for at least 3 weeks to allow this slow-growing bacteria to enter log phase.

4. To prepare the inoculum, 50-mL cultures of the bacterial strains are harvested at $2500 \times g$ for 25 min, washed with sterile saline and resuspended in saline at an optical density (OD_{540nm}) of 1.0.

5. Before infection of ferrets, 1 mL of each culture (from **step 4**) is mixed with 1 mL of feed slurry (*see* **Note 4**) and offered to each ferret in a sterile 3-mL syringe (*see* **Note 2**).

3.4.2 Infection

1. The ferrets are separated into the treatment groups (*see* **Note 5**), and inoculated with 10^8 CFU per animal of wild-type MAP or 10^8 CFU of vaccine strains (*see* **Notes 6** and **7**). When conducting challenge studies, the ferrets are infected with wild-type MAP (10^8 CFU) at 4 weeks post vaccination.

2. The colony count is based on actual colonies observed after plating on 7H11 agar supplemented with 0.5% glycerol, 10% OADC, 0.05% Tween® 80 and 2μg/mL Mycobactin J. No antibiotics are added to these plates.

3. The plates were incubated at 37 °C for 6 weeks (*see* **Note 8**).

4. After infection, the animals are monitored for a few hours to ensure there is no acute reaction.

5. Fecal samples from each cage are collected at 2, 24, and 48 h post-infection.

6. The animals are weighed every week by placing the cage on a scale.

7. At week 1, 3, and 6 post-infection blood is collected from the cranial vena cava into heparinized 4-mL tubes. Collected blood samples are used for serum preparation as well as whole blood stimulation to measure IFN-γ responses.

3.4.3   IFN-γ Assay

1. To assess IFN-γ responses in ferrets, whole blood is stimulated with the following antigens: Johnin PPD, bovis PPD and avium PPD.

2. Briefly, whole blood is collected in heparin collection tubes, and 250μL was added to each well of a 96 well plate.

3. Each of the PPD antigens is used at 5μg/mL to stimulate for 18–24 h at 37 °C with 5% CO_2.

4. After 24 h, plasma is collected by harvesting and removing red cells/platelets at $400 \times g$ for 10 min and the remaining plasma is stored at −80 °C until analysis by ELISA.

5. Ferret IFN-γ is detected by ELISA following MabTech ELISA kit instructions.

3.4.4   Serum ELISA Assay for Antibody Production

1. Serum ELISA is conducted using IDEXX ELISA kits for Johne's disease.

2. Precoated plates from the kit are blocked and ferret serum samples are diluted 1:200.

3. Plates are washed and processed for antibody binding detection according to manufacturer's recommendations.

4. Assessment parameters are used according to the manufacturer's recommendations (>0.1 is positive).

3.4.5   Skin Test Assay

1. For the skin test, an intradermal injection of the Johnin PPD antigens are conducted at weeks 0, 4, and 8 post-infection.

2. Tuberculin units (TU) of the PPD antigen are calculated and prepared as follows:
   (a) Antigen is diluted in PBS to obtain a 20μg/mL working stock and 100μL (2μg/0.1 mL/ferret = 100 TU or IU) are intradermally injected into each ferret.
   (b) Diluted antigen is placed in 1-mL tuberculin syringes until ready for injections.

3. The two flanks of the ferrets are shaved with an electric clipper and a disposable razor.

4. The shaved area is cleaned with 70% EtOH and 0.1 mL intradermal injections of PBS and *M. bovis* PPD are instilled on one flank, while *M. avium* PPD and Johnin PPD are injected on the other flank.

5. The concentration of PPD used can range from 100 to 400 TU. The injection sites are marked with a Sharpie® pen for ease of locating the injections sites for later measurements.

6. The erythema and induration reactions on the skin are measured at 24, 48 and 72 h post-infection using calipers (*see* **Notes 9–11**).

3.4.6 Fecal Culture

1. For the fecal culture assay, samples from each cage tray are collected and stored at −80 °C every week (*see* **Note 12**). When all samples have been collected, they are then processed in parallel by decontamination and cultured on HEKK tubes for 6 weeks at 37 °C.

2. Feces are weighed (0.5 g) in a small disposable weigh boat and then added to a 50-mL conical tube containing 25-mL of sterile dH$_2$O.

3. Tubes are affixed onto an Eberbach shaker, secured with screws, and shaken on high for 30 min to disperse fecal clumps.

4. Tubes are removed from the shaker and placed upright in a rack for 30 min to allow particulates to settle.

5. The settled material is decontaminated using the NADC method as described previously [12] and consists of a 0.9% hexadecylpyridinium chloride (HPC) decontamination step.

6. Material is plated in 0.1-mL volumes on HEYM slants with antibiotics (ANV).

3.4.7 PCR Assay

1. PCR amplification is performed on fecal slurries (from **step 4** immediately above) using the following conditions (*see* **Note 13**).
   (a) Denaturation: 94 °C for 5 min.
   (b) 30 cycles of 94 °C for 30 s, 65 °C for 1 min, 70 °C for 30 s.
   (c) Final extension: 70 °C for 5 min.

2. Amplified products are stored at 4 °C until gel electrophoresis or analyzed directly for C_t values if conducting real time PCR. A C_t value above the negative control value is considered a positive result.

3.5 Experimental Endpoint

For this model, we used a 20-week post-infection timeline; however, depending on strains and dose scheduling, the experimental end point may be extended for additional weeks. At 20 weeks post-infection, the animals are anesthetized with a 2× dose of 60 mg/kg ketamine and 0.04 mg/kg dexmedetomidine. Once, there is no pedal reflex to pinching, the anaesthetized animals are

exsanguinated by collecting blood directly from the heart (~10–20 mL). Once, the heart stops, the thoracic cavity is immediately opened. This serves as the gateway for collecting tissues including liver and mesenteric lymph nodes. The spleen is also collected to prepare splenocytes to be stimulated with media, MAP extract, PPD, and PHA. Intestinal sections were processed for histopathology. Also, at the end point, blood is collected for serum preparation and PPD stimulation, lungs, intestines for CFU and histopathology, feces for CFU and PCR. At euthanasia feces was collected from the terminal colon from each animal.

3.5.1 Histopathology on Ferret Tissues

1. Histopathology is performed on the tissues collected at the experimental end point.

2. The small and large intestines from each ferret are fixed in 10% buffered formalin at room temperature. Tissues are fixed for approximately 7 days.

3. Three small cross-sections of the duodenum, jejunum and ileum are cut from the fixed intestinal tissue and placed in a tissue cassette, which is then immersed in formaldehyde until ready to stain.

4. Five-micron sections are then stained with hematoxylin and eosin and the slides should be read by a board-certified pathologist.

When the ferrets are handled in this manner, one can expect to observe normally active and healthy appearing animals throughout the course of the study. Ferrets should lose weight after week 16 post-infection. However, activated Peyer's patches and increased villi expansion in the intestinal tissues may occur with corresponding weight loss. Also, a serum antibody response should develop by week 13, but an IFN-γ response from cells stimulated with PPD or sonicated lysates of MAP may not be detected in whole blood or splenocytes before the end of the study period. CFUs on fecal culture may appear after week 9.

In summary, these methods can be applied to assess vaccine candidates in a ferret model of Johne's disease, but these methods could also serve as a framework for use in other bacterial systems. While the scope of this chapter covers only the infection and handling of ferrets with MAP, additional supplemental protocols can now be developed to use ferrets for vaccine-challenge trials.

4   Notes

1. We recommend purchasing the ferrets from Triple F Farms, Inc., Gillett, PA, 16925, USA. This source provides healthy animals in good condition and is widely used in infection studies.

2. Initially, the ferrets may resist eating from the syringe. However, after a day or two they will start eating through the syringe without any waste. This is important to get a consistent inoculum in all the ferrets.

3. For preparation of the Oxbow feed slurry, 0.5–1 tsp. of feed was stirred in 1 mL water to make a slurry.

4. Mycobacteria readily form clumps in culture. Therefore, it is important to mix the culture-feed inoculum thoroughly with the syringe or conduct a brief 15 s sonication at a 50% duty cycle. Ferrets are fed orally through a 3-mL syringe barrel immediately after preparation.

5. Cages should be arranged such that infected ferrets are well separated from uninfected ferrets. The minimum separation recommended is across a 10 ft-room.

6. It is best to prepare the infection doses on the same day they will be used. If this is not possible then keep the loaded syringes at 4 °C and administer within 24 h. If stored at 4 °C for any length of time, invert the syringe several times to remix the settled slurry.

7. With the ferrets already trained to take these types of feedings through the syringe, they will consume the entire slurry without any loss of inoculum.

8. Patience is essential during the infection as the slow growth of the mycobacteria takes at least 6 weeks to manifest colonies on agar plate and even longer to observe disease signs in the ferrets.

9. Calipers are a mathematical tool used to measure distance. In this case the tool is used to measure the size of the inflammation (erythema) nodule on the skin as a result of the PPD injection.

10. The animal can be lightly anesthetized with Isoflurane to prevent excessive movement while measuring the reaction.

11. Ferrets might not respond well in skin tests and therefore it is not recommended to increase the amount and frequency of intradermal injections as this may result in positive tests in the sham-inoculated animals.

12. The feces are collected from the cage pan and therefore is representative of all the cage mates and not individual animals. The pan is also checked for sign of blood during fecal collection.

13. The target for PCR amplification is the MAP-specific IS*900* element using 200 picomoles of primers 5′-CCGCTAATTGA GAGATGCGATTGG-3′ and 5′-AATCAACTCCAGCAG CA GCGCGGCCTCG-3′.

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<div style="text-align: right">**Chapter 6**</div>

Recombinant *Escherichia coli* Cell Lysates as a Low-Cost Alternative for Vaccines Against Veterinary Clostridial Diseases

Clóvis Moreira Jr., Gustavo Marçal Schmidt Garcia Moreira, Rafael Amaral Donassolo, Marcos Roberto Alves Ferreira, Mariliana Luiza Ferreira Alves, Rafael Rodrigues Rodrigues, and Fabricio Rochedo Conceição

Abstract

This chapter describes a practical, industry-friendly, and efficient vaccine protocol based on the use of *Escherichia coli* cell fractions (inclusion bodies or cell lysate supernatant) containing the recombinant antigen. This approach was characterized and evaluated in laboratory and farm animals by the seroneutralization assay in mice, thereby showing to be an excellent alternative to induce a protective immune response against clostridial diseases.

Key words Clostridiosis, Recombinant vaccines, Recombinant antigens, Inclusion bodies, Cell lysate supernatant

1 Introduction

Pathogenic Clostridia produce a variety of potent toxins responsible for neurotoxic, histotoxic, or enterotoxic pathologies in both domestic and wild animals [1]. Commercial polyvalent clostridial vaccines are based on formaldehyde-inactivated toxins or bacteria and are currently the main approach to control these diseases. Although efficient, these vaccines present a time-consuming production process and pose safety risks. As alternative, experimental recombinant vaccines have been successfully evaluated in many animal species [2–5]. However, the production of purified recombinant antigens may represent a cost increase to the product that is not attractive for the veterinary industry context since it requires additional steps, such as antigen solubilization and refolding.
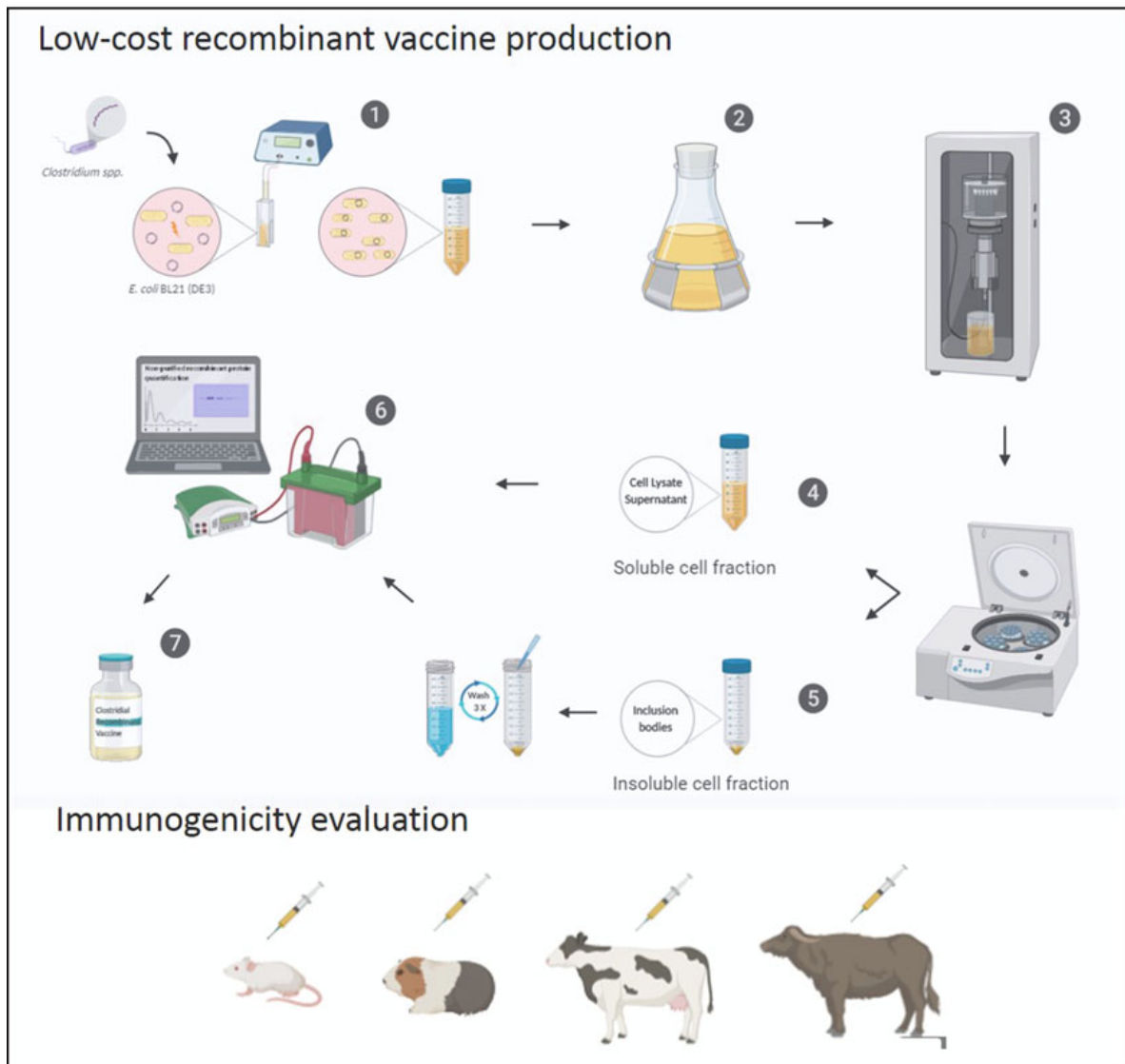
Fig. 1 Scheme describing a step-by-step the production of recombinant *E. coli* cell lysate to be used as a vaccine against clostridial diseases in animals

To overcome this cost problem, immunization of animals with non-purified recombinant antigens, which can be easily obtained from *E. coli* cell lysis and further use of its cellular fractions (i.e., inclusion bodies or supernatant of cell lysate) containing the recombinant antigen [4–6].

The production protocol to obtain these recombinant *E. coli* cell lysate fractions depict a practical, industry-friendly, and efficient vaccine formulation. This process involves seven simple steps (Fig. 1): (1) Transformation of *E. coli* BL21 (DE3) strain with a plasmid vector containing T7 promoter and a gene of *Clostridium* spp.; (2) Expression of target protein in *E. coli*; (3) Cell disruption and processing of *E. coli* cell lysates; (4) Preparation of soluble cell fraction; (5) Preparation of insoluble cell fraction; (6) Analysis by

SDS-PAGE/western blot and protein quantification; and (7) Vaccine formulation and immunogenicity evaluation.

This simple production strategy allows the reduction in the production timeframe as well as in the risks involved during production of native clostridial vaccines, once it usually involves recombinant, nontoxic fragments of the toxins. Experimental vaccines produced by this method have been evaluated in model and farm animals via seroneutralization assay in mice, showing to be a promising alternative to induce protective immune response.

2 Materials

2.1 Strain and Plasmids

1. *E. coli* strain BL21 (DE3) (Invitrogen, USA).
2. Expression vector containing a gene of interest from *Clostridium* spp.

2.2 Transformation, Expression, and Processing of E. coli Recombinant Proteins

1. $CaCl_2$ 100 mM.
2. Luria Bertani medium broth (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl to 900 mL of distilled water), homogenizer under orbital agitation e adjust to 1 L with distilled H_2O. For solid LB add 15 g agar in broth. Sterilize by autoclaving at 121 °C for 20 min and store.
3. Kanamycin (100 μg/mL).
4. Isopropyl β-D-1-thiogalactopiranoside (IPTG) with a final concentration of 1 M.
5. Electroporator: Bio-Rad Gene Pulser® II (Bio-Rad, USA).
6. Electroporation cuvette of 0.1 cm width, 1 cm length.
7. WPA CO8000 Cell density meter (Biochrom, UK).
8. Excella E24 Incubator shaker.
9. ThermoStat C Smartblocks™ 1.5 mL.
10. Ultrasonic processor VCX-500.
11. Refrigerated Centrifuge.
12. Cell Wash buffer (add 29.2 g NaCl, 2.34 g NaH_2PO_4, and 0.68 g imidazole in 800 mL of distilled water), adjust pH to 8.0 and make up to 1 L using distilled water. Filter the solution with 0.45 μm membranes.
13. Cell Lysis buffer (add 10 mg/mL lysozyme and 100 mM PMSF in Cell Wash buffer).

2.3 Polyacrylamide Gel Electrophoresis and Western Blotting

1. SDS-PAGE loading buffer 4×: Mix 4 mL glycerol, 2.4 mL Tris–HCl (1 M pH 6.8), 0.8 g SDS, 0.5 mL of β-mercaptoethanol and 4 mg bromophenol blue complete the final volume to 9.5 mL with distilled water. Store at −20 °C.

2. SDS-PAGE running buffer 5×: In 800 mL of distilled add 15.1 g Tris, 94.1 g glycine, and 5 g SDS. Mix and adjust pH to 8.3, complete the volume to 1 L. Store at 4 °C.

3. Coomassie Blue staining solution: add 1 g of Coomassie Brilliant Blue R-250 in 400 mL of distilled water, 500 mL of methanol and 100 mL of glacial acetic acid. Shake and filter using a paper filter.

4. Destaining solution: Mix 500 mL of distilled water, 400 mL of methanol and 100 mL of glacial acetic acid.

5. Gel SDS-PAGE 12%: Add 3.3 mL of H_2O, 4.0 mL of acrylamide/bis-acrylamide (30%/0.8% w/v), 2.5 mL of Tris–HCl (1.5 M pH 8.8) 0.01 mL of 10% (w/v) SDS; 0.01 mL 10% (w/v) of ammonium persulfate, 0.003 mL of TEMED. Cast gel within a 7.25 cm × 10 cm × 1.5 mm gel cassette support 5 mL of solution each. For stacking solution gel mix 1.4 mL of H_2O, 0.33 mL of acrylamide/bis-acrylamide (30%/0.8% w/v), 0.25 mL Tris–HCl (0.5 M pH 6.8), 0.02 mL of 10% (w/v) SDS, 0.02 mL ammonium persulfate, 0.002 mL of TEMED. One mL of stacking solution is enough for each gel.

6. Transfer buffer: In 700 mL of deionized water add 3 g Tris, 14.4 g glycine and 200 mL of methanol. Adjust the pH to 8.3 and make up the final volume to 1 L with deionized water. Store to 4 °C.

7. Ponceau S solution: Add 0.5 g of Ponceau in 0.1 mL acetic acid and make up to 100 mL using distilled water.

8. Nitrocellulose membrane.

9. Phosphate buffer saline (10×): Weigh 81.82 g of NaCl, 1.89 g of KCl, 1.91 g of KH_2PO_4, 28.62 g of $Na_2HPO_4.12H_2O$ in 700 mL of distilled water. Adjust the pH to 7.4 and complete to volume to 1 L. Sterilize by autoclaving at 121 °C for 20 min and store. The working solution is 1×.

10. Phosphate buffer saline containing Tween-20 (PBS-T): Add 0.05% Tween-20 in PBS 1×.

11. Blocking solution: Add 5% (w/v) skimmed milk powder in PBS-T 1×.

12. Monoclonal antibody anti-6xHis IgG.

13. Western blot substrate solution: Add 0.006 g of 3,3′- Diaminobenzidine tetrahydrochloride (DAB) in 9 mL Tris–HCl (50 mM), 1 mL of 0.3% $NiSO_4$ and 10 μL H_2O_2.

14. Amersham ECL Rainbow Marker—Full range.

15. Mini Trans-Blot® Cell.

16. Mini-PROTEAN Tetra cell.

17. PowerPac High-Current Power Supply.

2.4 Antigen Quantification	1. Pierce™ BCA Protein Assay Kit.
	2. Image Lab™ software (Bio-Rad, USA), CLIQS gel image analysis software (TotalLab, UK) or similar.
2.5 Vaccine Formulation and Immunogenicity Evaluation	1. Aluminum hydroxide.
	2. Thioglycollate broth: Weigh 29 g of Thioglycollate medium in 1 L of distilled water. Autoclave at 121 °C for 15 min.
	3. Sabouraud broth: Suspend 30 g of Sabouraud broth, dissolve in distilled water to a final volume of 1 L and adjust the pH to 5.6. Autoclave at 121 °C for 15 min.
	4. Standard toxins and antitoxins: Derived from institutions such as LANAGRO/MAPA (Brazil), NIBSC (UK), USDA (USA), or another competent organ or company.

3 Methods

***3.1 Transformation and Storage* E. coli BL21 (DE3) with Plasmid/Gene-of-Interest Vector**	1. Prepare an *E. coli* strain BL21 (DE3) culture by adding 10 mL LB into a 50-mL tube, and incubate at 37 °C, 200 rpm, for 16 h.
	2. Streak the grown cells onto a LB-agar plate and incubate at 37 °C for 16 h (*see* **Note 1**).
	3. Add 100 μL of $CaCl_2$ (100 mM) on a 1.5-mL tube and add 1–2 colonies of the grown *E. coli* BL21 (DE3).
	4. Add 1–5 μL of the recombinant plasmid (1–100 ng of DNA) and mix well. As a negative control of transformation use 1–5 μL of the pUC18 in the same conditions.
	5. Incubate on ice for 5 min.
	6. To promote heat-shock (*see* **Note 2**), incubate the 1.5-mL tube on Smartblocks™ at 42 °C for 45 s and quickly place it back on the ice for 5 min.
	7. Immediately add 1 mL of LB medium and incubate the cultures for 1 h at 37 °C under 150 rpm.
	8. Transfer 1 mL of each culture to a 50-mL flask with 9 mL of LB medium containing 100 μg/mL kanamycin. Incubate the cultures for 16 h at 30 °C under 150 rpm.
	9. Inoculate 10 mL of LB medium containing 100 μg/mL kanamycin with 100 μL of overnight culture.
	10. Incubate on shaker (150 rpm, 37 °C) for 1–3 h measuring the OD_{600} of culture until reaching the mid-log phase of growth ($OD_{600} = 0.6$–0.8).
	11. Add 10 mL of LB medium containing 20% (v/v) glycerol to 10 mL of culture at $OD_{600} = 0.6$–0.8.
	12. Distribute aliquots of 1 mL in cryotubes (this will be called "stock culture") and store at −20 or −80 °C.

3.2 Expression of Target Protein in E. coli Cell Culture

1. Dilute 1 mL of the stock culture into 50 mL of LB medium with 50 μL kanamycin 100 mg/mL in a 200-mL shake flask and incubate 16 h at 30 °C under 150 rpm.

2. Measure the OD_{600} of the culture and dilute a necessary volume mL to obtain a 0.1 OD_{600} in a 2-L shake flask containing 450 mL of LB medium with kanamycin 100 mg/mL (*see* **Note 3**).

3. Grow the cells under the same conditions (150 rpm, 37 °C) until it reaches the mid-log phase of growth (OD_{600} = 0.6–0.8). This should take about 2–3 h.

4. Add IPTG to a final concentration of 1 mM to induce expression of the target protein for 3–5 h in the same incubate conditions.

5. Collect 1 mL of the *E. coli* culture post-induction of recombinant protein (**step 4**) and centrifuge (10,000 × *g*, 2 min). Discard the supernatant and add 80 μL of wash buffer and 20 μl of loading buffer. Boil (10 min, 100 °C). Perform SDS-PAGE and western blot analyses.

6. Centrifuge the remaining culture (10,000 × *g*, 15 min, 4 °C), discard the supernatant, and store the pellet at −20 °C.

3.3 Preparation of Soluble Cell Fraction

1. Suspend the pellet (*see* Subheading 3.2, **step 6**) of the *E. coli* culture post-induction with 25 mL of cell lysis buffer.

2. Incubate the suspension for 1 h at 37 °C for lysozyme activity.

3. Incubate on ice for 20 min and transfer to an ultrasonic processor to sonicate (80 Hz) the suspension seven times for 30 s, with 15 s interval between each sonication.

4. Centrifuge (10,000 × *g*, 15 min, 4 °C) the tubes, transfer the supernatant to a new tube, and store the *E. coli* soluble cell fraction at 2–8 °C.

5. Collect 80 μL and add 20 μL of SDS-PAGE loading buffer 5×. Boil the sample (10 min, 100 °C), and store at −20 °C until it is used to perform SDS-PAGE and western blot analyses.

3.4 Preparation of Insoluble Cell Fraction

1. Suspend the pellet (*see* Subheading 3.2, **step 6**) of the *E. coli* culture post-induction of recombinant protein with 25 mL of cell lysis buffer.

2. Maintain the suspension for 1 h at 37 °C.

3. Put the suspension on ice for 20 min and transfer onto ultrasonic processor to sonicate (80 Hz) the suspension seven times for 30 s with 15 s of an interval between each sonication.

4. Centrifuge (10,000 × *g*, 15 min, 4 °C). Discard the supernatant and resuspend the pellet using 25 mL wash buffer.

5. Repeat **step 4** twice for washing the inclusion bodies.

6. Store the *E. coli* insoluble cell fraction containing the inclusion bodies at 2–8 °C.

7. Collect 80 μL and add 20 μL of SDS-PAGE loading buffer 5×. Boil the sample (10 min, 100 °C), and store at −20 °C until it is used to perform SDS-PAGE and western blot analyses.

3.5 SDS-PAGE and Western Blotting Analyses

1. Use 12% polyacrylamide gels to run the samples. Load 10 μL of each collected sample and 5 μL of protein molecular weight marker (10–200 kDa) per well on the gel.

2. Add 1× running buffer until filling the electrophoresis apparatus. Run for 100 V for approximately 2 h.

3. Place gel in a staining tray with 50 mL of Coomassie blue staining (or enough to completely coves gel) and shake on a rocker for at least 3 h at room temperature.

4. Remove the staining solution and add 100 mL of the destaining solution. Keep shaking until the bands can be seen, and the empty parts of the gel are transparent.

5. Transfer proteins to a nitrocellulose membrane by placing the gel in contact with the membrane between filter papers and in under the Mini Trans-Blot® Cell, run at 4 °C for 60 min at 100 V.

6. Remove the membrane and add 20 mL 0.1% Ponceau S solution to check the transfer efficiency. Wash the membrane on a rocker with distilled water for 5 min.

7. Incubate the membrane in a tray with PBS-T containing 5% (w/v) skimmed milk powder for 1 h.

8. Remove the liquid and wash three times using PBS-T.

9. Add monoclonal antibody anti-6xHis IgG diluted (1:10,000) in PBS-T for 1 h, and repeat the washing.

10. Add substrate solution to develop the reaction for maximum 10 min.

3.6 Antigen Quantification

1. Perform SDS-PAGE by adding 10 μL per well of each previously prepared sample for antigen quantification. Load the sample of non-transformed *E. coli* as a negative control together with the samples of *E. coli* post-induction of recombinant protein soluble cell fraction, and insoluble cell fraction.

2. Load a standard calibration curve of recombinant purified protein or BSA (Pierce™ BCA Protein Assay Kit) to obtain a range of 0.5–5 μg in the same gel of the samples to be quantified (*see* **Note 4**).

3. Use CLIQS gel image analysis software (TotalLab, UK), or similar, to build a standard curve based on the recombinant protein or BSA, which were loaded using defined amounts.
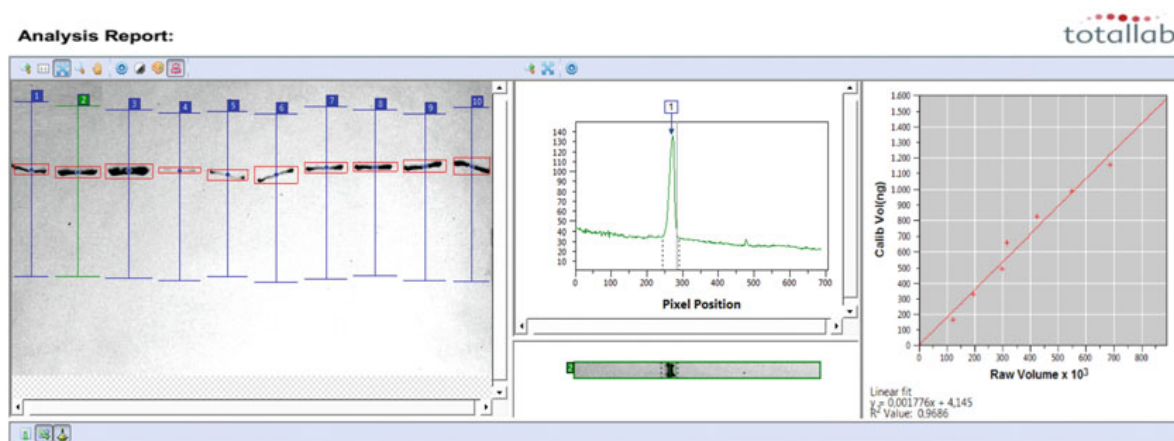
Fig. 2 Protein quantification using CLIQS gel image analysis software (TotalLab, UK). Recombinant proteins in *E. coli* cell fractions are quantified based on a standard curve made with defined amounts of either a purified recombinant protein or BSA loaded on a gel. This method can be used for quantification via both SDS-PAGE or western blot

4. Quantify the amount of recombinant antigen on *E. coli* cell lysates fractions (Fig. 2, Lines 1–3) based on the values of the standard curve (Fig. 2, Lines 4–10) (Absolute quantify × band volume).

5. Alternatively, perform a western blot for quantification of recombinant protein from *E. coli* cell lysate fractions using the same purified protein on the calibration curve. Apply the image obtained in the CLIQS gel image analysis software to reduce the background interference of *E. coli* bands and obtain the cleanest image when compared to that obtained in SDS-PAGE (Fig. 2).

3.7 Vaccine Formulation

1. Add a sufficient volume of the suspension (either soluble or insoluble *E. coli* cell fractions) containing 100–400 μg/dose of recombinant antigen (*see* **Note 5**).

2. Add aluminum hydroxide adjuvant to a final concentration of 1.5–2.5% (w/v), and complete the volume with PBS buffer pH 7.4 (*see* **Note 6**).

3. Mix for 16–18 h at 25 °C under constant agitation for antigen adsorption with aluminum hydroxide.

4. Perform sterility test by culturing 1 mL of each formulation in 10 mL of thioglycolate and sabouraud broths and incubate at 37 °C and 25 °C, respectively. Check growth daily for 21 days by spectrophotometry.

5. Innocuity test is done by inoculating 5 mL of the formulation subcutaneously in two guinea pigs weighing 350–450 g (use two different application sites in each animal). Observe local reactions, signs of disease, or possible death for 7 days. If none of these adverse effects occur, the formulation is safe.

3.8 Sero-neutralization

1. Perform potency test with two groups of 10 guinea pigs. The first group receives 5 mL/dose of the recombinant *E. coli* cell fraction prepared as described previously; and the second receives 5 mL/dose of PBS mixed with 1.5–2.5% (w/v) aluminum hydroxide (negative control). All animals are vaccinated subcutaneously in a two-dose scheme on days zero and 21.

2. On day 42, perform bleeding by cardiac puncture, and separate the sera by centrifuging the blood ($3000 \times g$, 15 min). Store at 4 °C until use (*see* **Note 7**).

3. For each group, make three sera pools: Pool A is made by mixing 600 µL of 5 sera; pool B is the mix of the other 5; And pool AB is made the mix of 1 mL of pool A and B.

4. Mix 1 mL of standardized native toxin (1 L+/mL) with 1 mL of each dilutions (e.g., 1:100, 1:50, 1:10, 1:5, 1:2) of the animal pooled sera (A, B, and AB). Make the same dilutions with standard antitoxin as a positive control.

5. Incubate each mixed sample at 37 °C for 1 h.

6. Inoculate ten Swiss Webster mice weighing 18–22 g with 0.2 mL intravenously with each mixed sample.

7. Observe animals for survival during 72 h.

8. The survival information is used to calculate the IC_{50} and measure the results in international units per mL (IU/mL). The number of international units is the number of the first serum dilution in which no injected mice survive.

4 Notes

1. There is also the possibility to prepare chemically competent cells for direct transformation from cells stored at −80 °C. However, the procedure described in this chapter avoids the direct use of frozen cells for protein expression. Instead, it employs the preparation of fresh cells for heat-shock, also avoiding the use of frozen, transformed *E. coli* BL21(DE3), what might lead to problems in expression after long-term storage. Thus, the presented method implies that the plasmid construct should be stored either as purified DNA, or in a DNA-replicating *E. coli* strain to be extracted when needed.

2. Chemical transformation (heat-shock) and electroporation are the two most widely used methods, which are based on the permeability increase of the bacterial cell membrane to create pores. Thus, electroporation could also be performed in this step using commercial or in-house electrocompetent cells with the following procedure: add 50 µL of the cells into a electroporation cuvette and incubate on ice for 30 min. Add 1–3 µL

(1–5 ng) of the recombinant plasmid, place the cuvette in the electroporator, and perform the procedure (2.5 kV, 25 μF, and 200 Ω for 2–3 s). Immediately add 500 μL of LB medium to each transformation reaction and incubate at 37 °C, 200 rpm, for 1 h. The steps for both heat-shock and electroporation transformation are based on protocols previously described [7].

3. Use the equation $C1 \times V1 = C2 \times V2$ to calculate the volume of saturated *E. coli* cell culture (V1) needed to be transferred for 450 mL LB medium to reach 0.1 OD_{600}. For example: If measured OD_{600} is 1.6 would be necessary to transfer approximately 28.1 mL of cell culture to LB medium.

$$1.6 \times V1 = 0.1 \times 450$$

$$V1 = 28.1 \text{ mL}$$

4. The purified used as a standard for the calibration curve should be, preferably, the same molecule in the suspensions (cell lysate, inclusion bodies, or cell lysate supernatant). This allows the quantification by either SDS-PAGE or Immunoblot. Alternatively, quantification can be performed by SDS-PAGE gel, stained with Coomassie blue, by loading different known amounts of BSA.

5. Depending on the antigen solubility, use the preparation of the soluble or insoluble cell fraction in the vaccine formulation [6].

6. Mice, guinea pigs, and ruminants (bovine and buffaloes) have been successfully evaluated using 200 μg/mL in a final volume of 0.5 mL, 3 mL, and 5 mL, respectively, per dose [4–6, 8].

7. Alternatively, if an experiment was performed on other animal species other than guinea pigs, use the pooled sera from vaccinated animals of the same group to perform the seroneutralization in mice.

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Clostridium spp. Toxins: A Practical Guide for Expression and Characterization in *Escherichia coli*

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Abstract

Farm animals are frequently affected by a group of diseases with a rapid clinical course, caused by *Clostridium* spp. and immunization is essential to provide protection. However, the current manufacturing platform for these vaccines has disadvantages and the main alternative is the use of an expression system that uses *Escherichia coli* to obtain recombinant vaccine antigens. In this chapter we describe procedures for cloning, expression and characterization of recombinant toxins from *Clostridium* spp. produced in *E. coli* for veterinary vaccine applications.

Key words *Clostridium* spp., Toxins, Farm animals, *Escherichia coli*

1 Introduction

Farm animals such as cattle, sheep, pig, and poultry are often affected by pathogenic bacteria of the genus *Clostridium* spp. These are etiological agents that cause diseases such as botulism, tetanus, enterotoxemia, gas gangrene, necrotic enteritis, pseudo-membranous colitis, blackleg, bacillary hemoglobinuria, and more. The rapid clinical course of these infections often makes treatment unfeasible, culminating in death in the majority of cases. Thus, immunization of animals is the most viable and effective measure against them [1].

Currently, the main manufacturing platform of clostridial vaccines involves the cultivation of the pathogen and the production of toxins. The anaerobic metabolism and the fastidious characteristics of these microorganisms demand complex culture media, as well as specific fermentation conditions. Besides, the long time required

for toxin production (up to 6 days), together with the process of inactivating the toxins and bacteria (up to 10 days), further increase the costs of the process. The final product consists of either inactivated toxins (toxoids), or inactivated bacteria (bacterins), or both (bacterin-toxoid), depending on the *Clostridium* species used.

Considering the non-optimal conditions of *Clostridium* spp. culture, the search for alternative technologies for vaccine production have gained attention. The production platform that is mostly used as an alternative, employs recombinant proteins. In this context, an expression system that uses *Escherichia coli* to obtain recombinant vaccine antigens of *Clostridium* spp. not only increases yield, but also uses a non-pathogenic strain and reduces the time required to obtain the protein of interest [2]. In addition, by producing protective, less toxic (or non-toxic) regions of the toxin, it is possible to skip or diminish the long time required for toxin and bacterial inactivation [3].

In recent years, *E. coli* has been shown to provide advantages for *Clostridium* spp. toxins as a heterologous expression system. Thus, research over the development of recombinant low-cost, veterinary vaccines against clostridial species had received increased attention, as observed for *Clostridium botulinum*, *C. tetani*, *C. perfringens*, *C. chauvoei*, *C. septicum*, and *C. haemolyticum* [4].

Bearing in mind the above information, we describe here procedures for cloning, expression, and characterization of recombinant *Clostridium* spp. toxins produced in *Escherichia coli* for veterinary vaccine applications. Information regarding the antigens from *C. botulinum*, *C. tetani*, *C. perfringens*, *C. chauvoei*, *C. septicum*, *C. novyi*, *C. difficille*, and *C. haemolyticum* will be stated highlighting the main aspects to be considered for each of these species.

2   Materials

1. Luria–Bertani (LB) medium: Add about 700 mL of distilled water in a 1 L beaker. Weigh 10 g tryptone, 10 g NaCl, and 5 g yeast extract and transfer to the beaker. Mix and make up to 1 L with distilled water. Autoclave at 121 °C for 15 min.

2. DAB/H_2O_2 substrate solution: Mix 9 mL Tris–HCl 50 mM (pH 7.4), 1 mL $NiSO_4$ 0.3% and 6 mg DAB (3,3-′-diaminobenzidine) in a 15 mL tube. Incubate this solution with the blotting membrane after the addition of 10 μL of H_2O_2 30% (v/v).

3. SDS-PAGE loading buffer 5×: Mix 15 mL glycerol, 3 mL 10% SDS, 7.5 mL Tris—1 M HCl pH 6.8, 0.15 g bromophenol blue and 7.5 mL with distilled water in a 50 mL tube. In a chemical hood, add 2.1 mL of β-mercaptoethanol and dissolve

this solution in water bath. Make 1 mL aliquots in 1.5 mL tubes. Store at −20 °C

4. Binding buffer (BindBuff): 0.2 M NaH_2PO_4, 0.5 M NaCl and 10 mM imidazole, pH 8. For BindBuff-A, add 0.2 g *N*-lauroylsarcosine for each 100 mL before use. For BindBuff-B, add 0.4 g *N*-lauroylsarcosine for each 100 mL before use. For BindBuff-C, follow the same instructions for -A buffer, but add 6 M urea to the solution.

5. Coomassie Blue staining solution: Mix 400 mL of distilled water, 500 mL of methanol, and 100 mL of glacial acetic acid in a graduated cylinder and transfer the volume to a 1 L glass beaker. Weigh 1 g of Coomassie Brilliant Blue R-250 or G-250 and transfer to the beaker. Keep the solution under agitation with a magnetic bar for 5 min and filter using a paper filter placed in a funnel.

6. SDS-PAGE running buffer 10×: 30.3 g Tris, 144 g glycine and 10 g SDS. Mix and make up to 1 L with distilled water. Adjust pH to 8.3 with HCl. Dilute the buffer ten times by measuring 100 mL to a graduated cylinder more 900 mL of distilled water. The working solution is now 1×.

7. Destaining solution: 500 mL of distilled water, 400 mL of methanol, and 100 mL of glacial acetic acid. Mix in a graduated cylinder and store in a 1 L glass flask.

8. PBS 10×/PBS-T: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4. Dilute in 800 mL of distilled water, adjust pH to 6.8 and make up to 1 L. Dilute the buffer ten times by adding 100 mL to a graduated cylinder plus 900 mL of distilled water. The working solution is now 1×. Add 0.5 mL Tween 20 for each 1 L of PBS 1× to prepare PBS-T.

9. Transfer buffer: 2.9 g glycine, 9.8 g tris-base, 3.7 ml SDS 10% (w/v) and 200 mL of methanol. Mix and make up to 1 L with distilled water. Adjust pH to 8.3 with HCl.

10. Glycerol 50% (v/v): add 50 mL of pure glycerol (99% purity) to 50 mL of Milli-Q water. Autoclave at 121 °C for 15 min.

11. Carbonate-Bicarbonate buffer: 1.59 g Na_2CO_3, 2.93 NaHCO. Mix and make up to 1 L with Milli-Q water. Adjust pH to 9.6.

12. Mouse anti-6xHis antibody (Sigma-Aldrich) and goat anti-mouse HRP-conjugated (Sigma-Aldrich) in dilutions provided by the manufacturer.

13. OPD/H_2O_2 substrate solution: 28.4 g Na_2HPO_4; add Milli-Q water until 1 L. 21.01 g citric acid; add Milli-Q water until 1 L. Mix 1.16 mL of Na_2HPO_4 solution and 1.32 mL citrate solution, add 2.53 mL H_2O, 6 μL H_2O_2, and 2 mg OPD.

3   Methods

3.1   Cloning

1. The gene encoding the protein of interest can be designed and already ordered in an expression vector of preference.

2. Add 100 μL of electrocompetent *E. coli* cells (DH5α™ or TOP10 strains) to electroporation cuvettes.

3. Mix 10 ng of plasmid DNA and proceed to electroporation with the following conditions: 25 μF, 2.5 kv, 200 Ω, 5.0 ms.

4. Add 0.5 mL of LB medium to the cuvette, transfer the whole volume with cells to a 1.5-mL tube, and incubate 1 h at 37 °C.

5. Centrifuge the tube (1 min, 8000 × *g*) and discard supernatant until 100 μL of medium is left in the tube.

6. Spread the whole volume onto LB-agar containing the proper antibiotics, and incubate at 37 °C for 16 h.

7. Perform colony PCR to screen for colonies containing the recombinant plasmids.

8. Pick 2 to 3 colonies identified as positive, inoculate each in 10 mL of LB in a 50-mL tube, and grow it at 37 °C for 16 h.

9. Make glycerol stocks of each clone by adding one part of Glycerol 50% (v/v) and two parts of the bacterial culture into cryotubes.

10. Store the tubes at −80 °C.

11. Use the remaining culture for plasmid DNA extraction using kit (such as Mini Prep plasmid extraction Kit—GE Healthcare, UK), quantify extracted plasmids, and confirm purity on an agarose gel electrophoresis.

3.2   Protein Expression

1. Streak an *E. coli* BL21 (DE3) strain of preference onto LB-agar, and incubate at 37 °C for 16 h (*see* **Note 1**).

2. In a 1.5-mL tube, mix 100 μL of CaCl$_2$ 0.1 M, 200 ng of recombinant plasmid, and 3–5 colonies from the freshly grown plate.

3. Perform heat-shock transformation by incubating the tube on ice for 5 min, transfer it rapidly to 42 °C for 1 min, and transfer it back again to ice for 5 min.

4. Add 0.5 mL of LB in each tube and incubate at 37 °C for 1 h, under 200 rpm.

5. Transfer the transformed cells to 10 mL of LB containing the appropriate antibody in a 50 mL tube, and incubate at 37 °C for 16 h.

6. Add a certain volume of the culture to 200 mL of LB in a 1-L flask until OD$_{600}$ = 0.1.

7. Incubate the culture at 37 °C, 200 rpm, until $OD_{600} = 0.6$–0.8.

8. Separate 5 mL from the flask to a 50-mL tube (this is called "non-induced sample").

9. Add IPTG (isopropyl-β-D-1-thiogalactopyranoside) to a final concentration of 500 mM (this is called "induced sample"), and incubate both cultures at 37 °C for 3 h, under 150 rpm (*see* **Note 2**).

10. For SDS-PAGE sample preparation, collect 1 mL of both non-induced and induced sample, adjust to $OD_{600} = 0.65$, centrifuge ($8000 \times g$, 1 min), discard supernatant and suspend cells in 100 μL of SDS-PAGE loading buffer $1\times$ (*see* **Note 3**).

11. Centrifuge the remaining culture ($10{,}000 \times g$, 10 min, 4 °C), and store both the samples and culture pellet at -20 °C until use.

3.3 Characterization of the Recombinant Protein

1. Suspend the pellet stored at -20 °C from the expression described in the previous topic in 25 mL of BindBuff in a 50-mL tube.

2. Add 50 mg/mL of Lysozyme and incubate 1 h at 37 °C, under 200 rpm.

3. Incubate the tube in ice for 20 min and perform sonication using 5–8 times 20 s cycles, with 10 s interval and 60 kHz.

4. Centrifuge the lysed cells ($10{,}000 \times g$, 10 min, 4 °C) and save the supernatant (this is called "supernatant" (SN) of lysis). Use 75 μL of this fraction to prepare an SDS-PAGE sample.

5. Suspend the pellet in 25 mL of BindBuff-A and incubate the tube for 16 h at 4 °C on a rocker.

6. Centrifuge the sample ($16{,}000 \times g$, 10 min, 4 °C), save the supernatant, and prepare an SDS-PAGE sample with 75 μL of this fraction.

7. Repeat **steps 5** and **6** using BindBuff-B and -C for cell suspension.

8. Heat samples at 95 °C for 10 min and run SDS-PAGE gels using 10 μL per well. Each gel should be run twice: one for Coomassie staining, and another for western blot (*see* **Note 4**).

9. Stain one of the gels using Coomassie Blue R250 solution for at least 4 h at RT, and incubate in destaining solution until gel becomes transparent.

10. Transfer one of the gels to a nitrocellulose membrane using a Bio-Rad blot chamber and transfer buffer (1 h, 100 V or 18 h, 30 V).

11. Block the membrane in PBS-T containing 2% (w/v) skimmed milk powder for 16 h at 4 °C.

12. Throw the blocking solution away, and incubate the membrane with mouse anti-6xHis antibody (Sigma-Aldrich) for 1 h at RT, followed by goat anti-mouse HRP-conjugated (Sigma-Aldrich), also for 1 h at RT (wash the membrane three times with PBS-T after each incubation step) (*see* **Note 5**).

13. Develop the reaction using DAB/H_2O_2 substrate solution for 10 min and analyze both the gel and immunoblot in regards the yield (*see* **Note 6**).

14. Define the fraction in which the desired protein is contained and proceed with Ni-affinity purification using 1 mL immobilized columns (*see* **Note 7**). Buffers used for purification should follow the same composition of the one in which the protein is contained, with the difference being imidazol concentration should be increased for the washing Buffer (20 mM Imidazol), and elution buffer (0.5 M Imidazol).

15. Elute the protein in 0.5-mL fractions and check for the presence of the protein in each fraction with spectrophotometry at 280 nm and SDS-PAGE followed by Coomassie staining.

16. Pool the fractions containing detectable amount of protein and dialyze it against PBS using 1 L per 1 mL of purified protein (*see* **Note 7**).

17. Perform protein quantification using BCA™ Protein Assay (Pierce) following manufacturer's instructions.

3.4 Antigenicity Evaluation

1. Perform SDS-PAGE and western blot the same way as described in **steps 10** and **11** of the previous topic with 2 μg of purified antigen per well.

2. Dilute standard anti-toxin to 1 IU/mL in PBS-T with 2% (w/v) skimmed milk powder and add to the membrane as primary antibody for 16 h at 4 °C, followed by 1 h at RT (*see* **Note 8**). Wash membrane with PBS-T, three times of 5 min.

3. Dilute HRP-conjugated antibody specific to the species in which the standard anti-toxin was produced in PBS-T containing 2% (w/v) milk powder and add to the membrane. Wash membrane with PBS-T, three times of 5 min.

4. Develop the reaction in the same method as described in **step 13** of the previous topic.

5. Prepare an ELISA plate by coating 200 ng/well (100 μL) of the purified antigen diluted in Carbonate-Bicarbonate buffer.

6. Prepare 10 1:2 serial dilutions of the standard serum in PBS-T with 2% (w/v) skimmed milk powder, add 100 μL/well, and incubate 1 h at 37 °C. Wash the wells three times with 200 μL/well of PBS-T.

7. Add 100 µL/well of the same secondary antibody used in **step 3**, and incubate for 1 h at 37 °C. Wash the wells three times with 200 µL/well of PBS-T.

8. Develop the ELISA using OPD/H_2O_2 substrate solution for 15 min in the dark and stop reaction using 100 µL/well of 1 N H_2SO_4.

9. Calculate the EC50 of each of the antigens tested to determine the best antigenic proteins (*see* **Note 9**).

4   Notes

1. The used expression strain depends on the characteristics of the designed antigen. A vast catalogue of expression *E. coli* strains are available online from many different companies. Here, we suggest BL21 DE3, pLysS, Star, RP, or RIL.

2. For the induction, it is recommended to test and optimize two parameters: the used concentration of IPTG, and the post-induction temperature. For the IPTG, it is recommended to test final concentrations between 0.1 and 1 mM. The choice of a lower or higher concentration will depend on the expression level of the protein checked in later steps. If too much protein is expressed in a way that it harms functionality or solubility, for example, it is recommended to reduce the concentration or add glucose at a final concentration of 20 mM. If low expression level is noticed in initial experiments, it is worth to try higher IPTG concentrations for the expression. As for the temperature, the recommended protocol is to start with 37 °C for 3–5 h. If problems such as aggregation, multiple band patterns, or even degradation are noticed, it is recommended to reduce temperature to either 30 or 20 °C and increase induction time to around 16 h [5].

3. Use 80 µL wash buffer after addition of 6 M urea to suspend cells and 20 µL SDS-PAGE loading buffer 5× if protein is too much insoluble.

4. For the SDS-PAGE gel, it is recommended to run a protein ladder of preference. In addition, a His-tagged protein with pre-determined concentration can be loaded in different amounts on the gel (typically 0.25, 0.5, 0.75, 1, 1.5, and 2 µg in each well) in order to estimate the expression level of the construct. As to the gel interpretation, if no apparent difference between non-induced and induced sample is noticed, the whole procedure can be performed without the use of IPTG. However, if the resulting molecule is showing poor solubility or activity in biological assays, it is recommended to begin the culture of the expression strains with LB

supplemented with glucose 1% (w/v). Then, medium has to be changed for expression by centrifuging the cultures ($8000 \times g$, 1 min).

5. The images from the gel and immunoblot can be used for protein quantification using gel densitometry. Programs *ImageJ* (NIH) and *TotalLab quant* is recommended. The analysis should use the concentration curve of the His-tagged protein as reference for quantification.

6. It is likely that proteins show in more than one of the BindBuff fractions. However, one of the fractions has to be chosen for further protein characterization and use. Thus, it is recommended to prioritize the first buffers used, i.e., BindBuff should be the first option, followed by BindBuff-A, -B, and -C, respectively. The choice depends on the amount of protein present in each of the fractions, e.g., if a protein is present in BindBuff and BindBuff-A, but the latter contains the majority of the produced molecule, this can be considered the fraction of choice.

7. The dialysis protocol depends directly on the behavior of the antigens expressed and on the level of solubility of the designed proteins. The main goal of the dialysis is to reduce the amount of denaturing agent in the final preparation (i.e., NLS or Urea). Usually, these chemicals cannot be removed promptly and must have their concentrations gradually decreased. Thus, dialysis should be performed gradually, by adding 200 mL PBS hourly (overnight dialysis can also be considered) until 4–5 L is reached. Alternatively, inert detergents can also be added to the PBS in order to reduce chances of protein aggregation and precipitation, such as Tween 20 0.05% (v/v), or Triton X-100 0.05% (v/v).

8. Standard anti-toxin sera can be acquired for regulatory agencies for biological standards in different continents, such as LANAGRO/MAPA (Brazil), NIBSC (United Kingdom), USDA (USA), or other.

9. The ELISA for antigenic evaluation might have to be optimized for each respective antigen in regards to the amount of coated antigens. In addition, consider making more dilutions in case depletion is not reached.

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Part V

Vaccines for Veterinary Parasites

Chapter 8

Macrophage Stimulation as a Useful Approach for Immunoscreening of Potential Vaccine Candidates Against *Toxoplasma gondii* and *Neospora caninum* Infections

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Abstract

Toxoplasmosis and neosporosis are protozoan diseases that adversely affect the medical and additionally veterinary sectors, respectively. Toxoplasmosis is caused by *Toxoplasma gondii* which infects almost all warm-blooded animals including humans. While, neosporosis is caused by *Neospora caninum*, which induces infection in many animal species particularly in cattle. Currently, control measures for both infections are defective because of no effective vaccine or treatment. Macrophages constitute the first line of innate immunity, which contributes to the effective elimination of *T. gondii* or *N. caninum*. This action is mediated by IL-12, which is critical for the secretion of interferon gamma (IFN-γ). Successful vaccine candidates against both protozoan parasites should possess the ability to induce the cellular immune response and IFN-γ production. In this chapter, we will focus on an efficient immunological approach for discovery of potential vaccine candidates against above-mentioned parasites. Our previous studies revealed a strong correlation between vaccine antigens that enhanced the macrophage secretion of IL-12 and their efficacy as potential vaccine candidates in murine model. In case of *T. gondii*, peroxiredoxin 1 (TgPrx1) and peroxiredoxin 3 stimulated the production of IL-12 from murine peritoneal macrophages and conferred strong to moderate protection in C57BL/6 mice, respectively. At the same context, *Neospora* antigens of dense granule protein 6 (NcGRA6) and cyclophilin entrapped with oligo-mannose coated-liposomes stimulated macrophage IL-12 secretion and substantially protected immunized BALB/c mice. Therefore, we can deduce that macrophage stimulation evidenced in IL-12 production can be used as a useful approach for judgment of vaccine efficacy before further evaluation using in vivo experiments. Methods of vaccine preparation and macrophage stimulation will be fully described for TgPrx1 and NcGRA6 as potential vaccine candidates against toxoplasmosis and neosporosis, respectively.

Key words Vaccine, Macrophage, *Toxoplasma gondii*, *Neospora caninum*

1 Introduction

Toxoplasmosis and neosporosis are heteroxenous protozoan diseases responsible for substantial losses in medical and additionally veterinary sectors, respectively. Toxoplasmosis is caused by

Toxoplasma gondii which infects almost all warm-blooded animals, with considerable hazards in human, sheep, and pigs. Approximately a third of the world's human population has been found as seropositive to specific anti-*T. gondii* antibodies. Infection is primarily caused by ingestion of contaminated food or water with oocysts or by eating raw or undercooked meat containing tissue cysts. The disease is mostly asymptomatic in immunocompetent individuals or animals. Oppositely, in immunocompromised patients, primary infection or reactivation of latent disease might induce fatal consequences. In the same context, *T. gondii* infection during pregnancy may cause abortion or fetal anomalies [1, 2].

Neosporosis is caused by *Neospora caninum,* the intracellular apicomplexan parasite, can induce infection in many animal species particularly in cattle, sheep and dog. This parasite is similar to *T. gondii* in many phenotypic, genetic and immunological characteristics. Infection can be transmitted via two routes; orally via the ingestion of oocysts or tissue cysts, and vertically from an infected dam to the fetus by transplacental transmission. Abortion outbreaks and culling of infected animals are the major factors for the substantial financial burdens and losses in cattle industry [3, 4].

In general, immune cells are usually divided into two groups; T helper 1 (Th1) and T helper 2 (Th2) subpopulations depending on the type secreted cytokines. The Th1 cells secrete gamma Interferon (IFN-γ), Interleukin 2 (IL-2), IL-12, and Tumor Necrosis Factor-alpha (TNF-α) whereas the Th2 cells produce IL-4, IL-5, IL-10, and IL-13. Protective immunity against toxoplasmosis and neosporosis is predominantly attributed to a Th1 type of response and IFN-γ secretion [4–7]. The secretion of IFN-γ is induced by various immune cells as a feedback to IL-12 production from macrophages. IFN-γ has been reported as an essential mediator of resistance against *T. gondii* and *N. caninum*. IFN-γ has the potential to activate the macrophages to kill intracellular parasites and to stimulate cytotoxic T cells to destroy infected cells. However, antibodies also contribute to controlling the infection in case of *T. gondii* or *N. caninum* either by neutralizing the secreted antigens or restricting the parasite dissemination [4, 6–8].

Vaccine studies against *T. gondii* or *N. caninum* had been initially focused on using the live, live attenuated and killed tachyzoites. Nevertheless, their uses were restricted because of fears of resuming pathogenicity. Thus, recent trends of vaccine development have been shifted to vector-based or subunit vaccines. The recombinant protein is a type of subunit vaccine that proved its efficacy as vaccine antigens either alone or after formulation with adjuvant substance. High safety of recombinant vaccine antigens is an essential additional advantage against all other types of vaccine particularly if they provide long-term and potent immunoprotective efficacy [4, 7, 9–11].

Macrophages or monocytes are the first defense line for innate immune response against almost all pathogens. They also have the ability to mediate the adaptive or acquired immunity directly through antigen presentation or indirectly by secreting many effector molecules including cytokines. In which, in case of infection, macrophages have been reported to participate in the parasite killing through the phagocytosis or via the production of diverse kind of proinflammatory cytokines and effector molecules [12, 13]. As professional antigen presenting cells (APC), macrophages are critical for developing appropriate immune response against the vaccine antigens or those secreted from the parasite during infection. During such complicated process, a substantial number of immune effector molecules are produced [4, 7, 9–11].

In this chapter, we will focus on an efficient immunological approach for discovery of potential vaccine candidates against above-mentioned parasites. Our previous studies revealed a strong correlation between vaccine antigens that enhanced the macrophage secretion of IL-12 and their efficacy as potential vaccine candidates in murine model. In case of *T. gondii*, peroxiredoxin 1 (TgPrx1) [14], and TgPrx3 [15] stimulated the production of IL-12 from murine peritoneal macrophages and conferred strong and moderate protection in C57BL/6 mice, respectively. At the same context, *Neospora* dense granule protein 6 (NcGRA6) [16], and cyclophilin entrapped with oligo-mannose coated-liposomes (NcCyp-OML) [17] stimulated macrophage IL-12 secretion and substantially protected immunized BALB/c mice. Therefore, we can deduce that macrophage stimulation evidenced in IL-12 production can be used as a useful approach for judgment of vaccine efficacy before further evaluation using *in vivo* experiments. Materials, reagents and methods of protein expression of TgPrx1 and NcGRA6 and macrophage stimulation will be fully described hereinafter. Both TgPrx1 and NcGRA6 were expressed as glutathione *S*-transferase (GST) fusion proteins in the *E. coli* expression system.

2   Materials

2.1   Recombinant TgPrx1 Vaccine [14]

1. *T. gondii* PLK.
2. Vero cells (African green monkey kidney epithelial cells).
3. TRI reagent.
4. SuperScript first-strand synthesis system for reverse transcription RT-PCR (Invitrogen, Carlsbad, CA).
5. Agarose gel.
6. Nucleospin gel and PCR clean up kit (Macherey Nagel, Düren, Germany).
7. Eagle's minimum essential medium.

8. Fetal bovine serum.

9. Streptomycin–penicillin.

10. Cell scraper.

11. 5.0-μm pore filter.

12. QIAprep DNA extraction kit (Qiagen Hilden, Germany).

13. Isopropyl-1-thio B-D galactopyranoside.

14. Bovine serum albumin.

15. Ampicillin Sodium.

16. Lysozyme

17. Dithiothreitol (DTT).

18. Polyoxyethylene (10) Octypheny Ether (Triton X100).

19. Polyoxyethylene (20) Sorbitan Monolaurate (Tween 20).

20. pGEX-4T3 plasmid vector (Amersham Pharmacia Biotech, Madison, CA, USA).

21. *Escherichia coli* BL21(DE3) (New England BioLabs Inc., Ipswich, MA, USA).

22. LB agar (Invitrogen, Carlsbad, CA), (prepared by adding 32 g to 1 L of distilled water (DW) followed by autoclaving).

23. LB broth (Invitrogen, Carlsbad, CA), (prepared by adding 20 g to 1 L of DW followed by autoclaving).

24. Big Dye Terminator Cycle Sequencing Kit (AB Applied Biosystems, Carlsbad, CA, USA).

25. ABI PRISM3100 genetic analyzer (AB Applied Biosystems).

26. ApE gene sequence and analysis software (Wayne Davis, Utah, USA).

27. Glutathione-sepharose 4B beads.

28. L-Glutathione, reduced- powder.

29. Detoxi-Gel Endotoxin Removing Column.

30. 0.45-μm low-protein binding Supor1 membrane.

31. Limulus Amebocyte Lysate reagents (LAL) (Seikagaku Inc., Tokyo, Japan).

32. Coomassie Brilliant Blue R250.

33. *N, N, N, N* Tetramethylene ethylene diamine (TEMED; formula weight = 116.20).

34. Sodium dodecyl sulfate (SDS).

35. Ammonium persulfate (APS).

36. Acrylamide.

37. Methylene bis-acrylamide.

38. Low molecular weight marker calibration for SDS (LMW).

39. Bicinchoninic acid (BCA) protein assay kit.

40. Buffers
    (a) Phosphate-buffered saline, pH 7.2 (0.01 M PBS; NaCl 8 g, KCl 0.4 g, KH_2PO_4 0.4 g, $Na_2PO_4 \cdot 12H_2O$ 5.8 g) dissolved in 1800 mL DW followed by pH adjustment to pH 7.4 before making the solution up to 2000 mL. It can be stored at room temperature or at 4 °C.

    (b) Sonication buffer (TNE; 100 mM Tris–HCl [pH 8], 100 mM NaCl, 5 mM EDTA). It should be stored at 4 °C.

    (c) Elution buffer (100 mM Tris–HCl [pH 8], 100 mM NaCl, 5 mM EDTA, 20 mM reduced glutathione powder). It can be stored at 4 °C or at −30 °C.

2.2 NcGRA6 Vaccine [16]

1. *N. caninum* (strain Nc-1).
2. TRI reagent.
3. SuperScript first-strand synthesis system reverse transcription RT-PCR (Invitrogen).
4. Agarose gel.
5. Nucleospin gel and PCR clean up kit.
6. Vero cells.
7. EMEM.
8. RPMI-1640.
9. FBS.
10. Streptomycin–penicillin.
11. Cell scraper.
12. 5.0-μm pore filter.
13. QIAprep DNA extraction kit.
14. IPTG.
15. BSA.
16. Ampicillin Sodium.
17. Lysozyme.
18. DTT.
19. Triton X100.
20. Tween 20.
21. pGEX-4T1 plasmid vector (Amersham Pharmacia Biotech).
22. Ampicillin (100 μg/mL).
23. *E. coli* BL21 (DE3).
24. LB agar.
25. LB broth.

26. Big Dye (AB Applied Biosystems).

27. ABI PRISM3100 genetic analyzer (AB Applied Biosystems).

28. ApE gene sequence and analysis software (Wayne Davis, Utah, USA).

29. Glutathione-sepharose 4B beads.

30. L-Glutathione, reduced- powder.

31. Acrodisc® Units with Mustang® E Membrane.

32. 0.45-μm low-protein binding Supor1 membrane.

33. Coomassie Brilliant Blue R250.

34. TEMED.

35. SDS.

36. APS.

37. Acrylamide.

38. Methylene bis-acrylamide.

39. LMW.

40. BCA.

41. Buffers (as previously described).
    (a) Phosphate-buffered saline.
    (b) Sonication buffer TNE.
    (c) Elution buffer.

2.3 Macrophages Preparation and Stimulation [14, 16]

1. Female 7-weeks-old C57BL/6 and BALB/c mice.

2. BBL™ Brewer modified thioglycolate medium.

3. Dulbecco's modified Eagle's medium.

4. 40 μm nylon cell strainer.

5. Trypan blue.

6. Hemocytometer Neubauer improved.

7. 96-well microplate.

8. Lipopolysaccharide.

9. Polymixin B.

10. Mouse IL-12p40 cytokine ELISA kits.

11. Buffers and diluents
    (a) PBS, pH 7.2.
    (b) RBC lysis buffer (0.83% NH_4Cl, 0.01 M Tris–HCl [pH 7.2]).
    (c) Cytokine ELISA coating buffer- 0.2 M Sodium Phosphate pH 6.5 (Na_2HPO_4 12.49 g, NaH_2PO_4 15.47 g dissolved in 900 mL of DW followed by pH adjustment to 6.5 by adding HCl or NaOH before making the solution up to 1000 mL. It should be stored at 4 °C.

(d) Cytokine ELISA assay diluent-PBS with 10% heat-inactivated FBS, pH 7. It should be freshly prepared just before use.

(e) Cytokine ELISA washing buffer-PBS with 0.05% Tween-20 (PBST), pH 7.4, 1 mL of Tween 20 is mixed thoroughly with an amount of 1 L PBS as described previously (*see* Subheading 2.1, **item 40a**), using magnetic stirrer. It can be stored at room temperature or at 4 °C.

(f) Substrate solution: Tetramethylbenzidine (TMB), The BD pharmingenTM TMB substrate reagent set. It should be stored at 4 °C.

(g) Cytokine ELISA stop solution-2 N H_2SO_4 (100 mL of concentrated H_2SO_4 is diluted to 1000 mL DW on ice). It should be stored at 4 °C.

3 Methods

3.1 Preparation of Recombinant TgPrx1-GST Vaccine Antigen [14]

3.1.1 Gene Amplification and Cloning

1. Search for the coding sequence of *T. gondii* Prx1 gene (GenBank accession number, XM_002371315.1 corresponding to amino acid positions 1 to 197) from cDNA of *T. gondii* PLK strain with PCR using oligonucleotide primers that included a *Bam*HI site (underlined) in the forward primer 5′-TA GGA TCC ATG CCG GCC CCG ATG GTG TCT-3′ and an *Xho*I site (underlined) in the reverse primer 5′-AG CTC GAG TTA CTT GCT TCC GAG ATA CTC-3′.

2. Wash and scrape African green monkey kidney (Vero) cells infected with *T. gondii* tachyzoites (PLK strain).

3. Wash the parasites and host cell debris with cold PBS, resuspend the final pellet in cold PBS, and pass through a syringe fitted with a 27-gauge needle for five times.

4. Pass the parasites through a 5.0-μm pore filter, wash them thoroughly with PBS (10 mL), and then centrifuge at $300 \times g$ for 10 min.

5. Extract total RNA from the parasites using TRI reagent.

6. Conduct first-strand cDNA synthesis from total parasite RNA with a SuperScript® First-Strand Synthesis System for reverse transcription RT-PCR.

7. Amplify the coding sequence of the TgPrx1 gene using TgPrx1-specific oligonucleotide primers and cut the band from the agarose gel and extract DNA using PCR/Gel clean up kit (*see* **Note 1**).

8. Digest the PCR products with *Bam*HI and *Xho*I and insert it into the pGEX-4 T3 plasmid vector treated with the same restriction enzymes.

9. Transform the constructed plasmid into *Escherichia coli* BL21 (DE3), then plate on LB agar medium overnight at 37 °C.

10. Pick-up the colonies and suspend each in 5 mL LB broth medium and incubate at 37 °C for 6 h.

11. Apply PCR screening for positive clones using amplification primers, then extract the DNA of plasmid and check by restriction enzymes to confirm the successful insertion of *TgPrx1* gene (*see* **Note 2**).

12. Check DNA from plasmid construct by sequencing using a Big Dye Terminator Cycle Sequencing Kit, and an ABI PRISM3100 genetic analyzer.

13. Analyze the nucleotide sequence of the obtained clone against target sequence from GenBank with ApE gene sequence and analysis software.

3.1.2 Protein Expression and Purification Procedures of TgPrx1-GST

Day 1:

1. Prepare a seed from positive TgPrx1-GST clone by adding 20 μL from stock to 20 mL LB containing 50 μg/mL ampicillin, then incubate it in shaker at 37 °C for overnight.

Day 2:

2. Add 20 mL culture to 1 L of LB containing 50 μg/mL ampicillin and 2% ethanol, and then incubate it in shaker at 37 °C until OD 600 nm of the culture reaches 0.50–0.55.

3. Add 1 mM IPTG to the culture, and then incubate it in a shaker (130 rpm) at 37 °C for 12 h (*see* **Note 3**).

Day 3:

4. Harvest the *E. coli* pellet by aliquoting 1 L culture to four 250 mL bottles, and centrifuge the culture at 8000 × *g*, 30 min, 4 °C, and then supernatant is discarded and the pellet is dissolved by 5 mL TNE.

5. Add 500 μg/mL lysozyme, and 1% Triton X100 in PBS, and then rotate it at 4 °C for 6 h.

6. Sonicate the sample on ice four times, 2 min each, and then centrifuge it at 8000 × *g*, 30 min, 4 °C.

7. Collect the supernatant and incubate it with 1 mL of pre-washed glutathione-sepharose 4B beads with 1% Triton X100 in 10 mL PBS, and then rotate it for overnight at 4 °C.

Day 4:

8. Wash the beads containing the fused protein for five times as follows; first, PBS, second, 1% Triton X100 in PBS, and third to fifth, PBS.

9. Add 1 mL of elution buffer (GEB) to 1 mL beads then allow for rotation at 4 °C for 3 h.

10. Collect the eluate by centrifugation at 2000 × *g*, 10 min, 4 °C, and then filtrate by 0.45 μm filter.

11. Check the protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (*see* **Note 4**).

12. Dialyze the protein in 1 L PBS for 12 h.

Day 5:

13. Remove the endotoxin using Detoxi-Gel Endotoxin Removing Column.

Day 6

14. Measure the concentration of protein by BCA kit.

3.2 Preparation of rNcGRA6-GST Vaccine Antigen [16]

3.2.1 Gene Amplification and Cloning

1. Search for the coding sequence of *NcGRA6* gene (from toxo. db, gene ID: NCLIV_052880) corresponding to amino acid positions 43 to 154 and lacking amino acids 1–43 (signal peptide) and 155–172 (transmembrane domain).

2. *See* Subheading 3.1, **step 2**.

3. *See* Subheading 3.1, **step 3**.

4. *See* Subheading 3.1, **step 4**.

5. *See* Subheading 3.1, **step 5**.

6. *See* Subheading 3.1, **step 6**.

7. Amplify the coding sequence of the *NcGRA6* gene using *NcGRA6*-specific oligonucleotide primers. The primers included an *Eco*RI site (underlined) in the forward primer (5′-AT **GAA TTC** ATG GAT CCG GTT GAA TCC GTG GAG-3′) and an *Xho*I site (underlined) in the reverse primer (5′-AT **CTC GAG** CTA TCT GTG ACG TGC CTG CTG CCG-3′), and cut the band from the agarose gel and extract DNA using PCR/Gel clean up kit (*see* **Note 1**).

8. Digest the PCR products with *Eco*RI and *Xho*I and insert it into the pGEX-4T1 plasmid vector treated with the same restriction enzymes.

9. Transform the constructed plasmid into *E. coli* BL21(DE3), then plate on LB agar medium overnight at 37 °C.

10. Pick-up the colonies and suspend each in 5 mL LB broth medium and incubate at 37 °C for 6 h.

11. Apply PCR screening for positive clones using amplification primers, then extract the DNA of plasmid and check by restriction enzymes to confirm the successful insertion of *NcGRA6* gene (*see* **Note 2**).

12. Check the target DNA sequence in the plasmid construct using a Big Dye Terminator Cycle Sequencing Kit, and an ABI PRISM3100 genetic analyzer.

13. Analyze the nucleotide sequence of the obtained clone against target sequence from toxo.db with ApE gene sequence and analysis software.

3.2.2 Protein Expression and Purification of NcGRA6-GST

Day 1

1. Prepare a seed culture from the positive NcGRA6-GST clone by adding 5 μL from *E. coli* stock to 20 mL LB containing 50 μg/mL ampicillin, and incubate it at 37 °C overnight.

Day 2

2. Add the 20 mL culture to 1 L LB culture containing 50 μg/mL ampicillin and 2% ethanol, and then incubate the culture in the shaker at 37 °C.

3. When OD 600 nm of the culture reaches 0.6, add 0.1 mM IPTG, and incubate the culture again at 37 °C in a shaker (160 rpm) for 6 h (*see* **Note 3**).

Day 3

4. Harvest the culture by centrifugation at 8000 × *g*, 10 min, 4 °C (as described in details for TgPrx1 preparation, Subheading 3.1.2, **step 4**).

5. Suspend the pellet from 1 L culture with 20 mL sonication buffer containing 500 μg/mL lysozyme, and incubate it on ice for 30 min.

6. Add 1% Triton X100 in PBS, suspend and incubate it on ice for 1 h.

7. Sonicate it on ice for 2 min, two times and check the suspension until turned to clear.

8. Centrifuge at 8000 × *g*, 10 min, 4 °C.

9. Collect the supernatant and mix with glutathione-sepharose 4B beads (1.33 mL) stabilized by PBS, and then incubate it at room temperature for 30 min with mild rotation. For stabilization of 1 mL from the beads, add 10 mL PBS, and centrifuge at 830 × *g*, 5 min, 4 °C and then discard the supernatant (Repeat this step for three times).

10. Centrifuge at 1000 × *g*, 5 min, 4 °C and remove the supernatant.

11. Resuspend the beads with 10 mL of PBST (PBS with 0.05% Tween20)

12. Centrifuge at 1000 × *g*, 5 min, 4 °C. Remove the supernatant.

13. Repeat **steps 10** and **11** three times.

14. Remove all PBST, and resuspend the beads with 20 mL PBS Centrifuge at 1000 × *g*, 5 min, 4 °C. Remove the supernatant.

15. Repeat **step 14** two times.

16. Add 1 mL elution buffer to the beads, and incubate at room temperature for 10 min with mild rotation.

17. Centrifuge at 850 × *g*, 5 min, 4 °C.

18. Collect the supernatants (1 mL).

19. Repeat **steps 16–18** for three times and remove coarse particles using 0.45 μm filter.

20. Check the protein by SDS-PAGE (*see* **Note 4**).

21. Mix the supernatants containing NcGRA6-GST, and dialyze it in 1 L PBS or 10–12 h.

    Day 4

22. Remove endotoxin using Acrodisc® Units with Mustang® E Membrane.

23. Quantify the concentration using BCA kit.

3.3 Isolation and Propagation of Thioglycolate-Elicited Peritoneal Macrophages [14, 16]

1. Inject C57BL/6 (in case of TgPrx1) or BALB/c (in case of NcGRA6) female 8-weeks-old by 2 mL 4.05% BBL™ Brewer modified thioglycolate medium via intraperitoneal route (*see* **Note 5**).

2. Four days after injection, collect peritoneal macrophages through peritoneal washes of 5 mL cold PBS twice.

3. **Steps 1** and **2** must be applied under deep anesthesia with isoflurane and under complete aseptic condition.

4. Centrifuge the collected cells at 1300 × *g* for 10 min and suspend in RBC lysis buffer for 10 min at 37 °C to lysis red blood cells.

5. Centrifuge again as previous, discard supernatant by aspiration, and then suspend the pellet in DMEM.

6. Discard the medium and suspend the pellet in 3–5 mL DMEM medium and pass through a 40 μm nylon cell strainer.

7. Prepare an amount 20 μL of cell suspension for counting and mix with equal amount of trypan blue to exclude the nonviable cells and count using hemocytometer.

8. Adjust the total amount and cell number by DMEM to 1.5×10^6/mL, then add 200 μL to a 96-well microplate to get a suspension of 3×10^5 cells/well and incubate the plate at 37 °C in a 5% CO_2 incubator for 4 h, allowing the cells adherence to the bottom.

9. During that time, prepare the required antigens in addition to negative and positive controls (in case of TgPrx1; cells were treated with 1 ng/mL LPS as positive control, and test antigens (rTgPrx1-GST and rGST) by 10,100 nM and medium as negative control either in presence or absence of 1 μg/mL polymixin B) (*see* **Note 6**).

10. While in case of NcGRA6, macrophages were treated with culture medium alone (mock), recombinant proteins GST and NcGRA6-GST at 100, 1000, 2500, 5000 μg/mL, *N. caninum* lysate antigen (NLA) at 20 and 50 μg/mL, or LPS at 10 ng/mL in the presence or absence of 10 μg/mL polymixin B (*see* **Notes 7** and **8**).

11. Incubate all prepared samples (test antigens, control negative and positive) with and without polymixin B at 37 °C for 2 h.

12. At scheduled time, 4 h after incubation of macrophages and 2 h after incubation of stimulants with polymixin B, aspirate the medium in the wells to remove the floating cells, add 200 μL the indicated antigens and controls, then incubate for 20 h at 37 °C in a 5% CO_2 incubator.

13. In the following day, collect an amount of 150 μL from supernatant and check cytokine IL-12p40 production using cytokine ELISA kit (*see* **Note 9**).

4　Notes

1. Agarose gel 1% is prepared by adding 1 g to 100 mL 1× TAE (TAE 50×, Tris base 121 g, Acetic acid 28.55 mL, 0.5 M EDTA pH 8 50 mL). Complete melting using microwave, thorough mixing, and slow pouring are necessary to avoid the gels clumps and air bubbles formation which may interfere with band running. For cutting the band and PCR clean up from the gel, cut the band of DNA and transfer to 15 mL centrifuge tubes. Add binding buffer 1 or 2 mL/tube (until cover the gel), then incubate at 50 °C for 10 min. Transfer the solution into spin column tubes, then centrifuge the tube at 10,000 × g, 4 °C for 1 min. Discard flow-through (DNA target is binding with the filter). Transfer the remaining of DNA target to spin column tubes (Repeat this step until use all melted gel is used). Add 750 μL binding buffer/tube, centrifuged at 10,000 × g, 4 °C for 1 min. Discard flow-through and add 750 μL of wash buffer, the tubes were centrifuged at 10,000 × g, 4 °C for 1 min and repeat this step again. Discard flow-through and air dry centrifuged at 10,000 × g, 4 °C for 1 min. Place the spin column tubes on the new 1.5 mL tube, and then add 25 μL of

elution buffer. Keep tube for 1 min at room temperature. Centrifuge at 10,000 × g, 4 °C for 1 min, the DNA is eluted to the 1.5 mL tube and keep at −30 °C.

2. After growth of colonies on LB agar, these colonies can be checked by PCR screening using designed amplification primer (only 500 μL is collected in 1.5 mL tube, centrifuged at 3000 × g, 5 min at room temperature, then pellet suspended with 20 μL DW and exposed to heat shock at 95 °C for 5 min), or the endonuclease digestion recognition and sequencing from extracted plasmid DNA. In pilot expression, 5–10 colonies can be selected to perform preliminary check.

3. In case of *E. coli* culture preparation, add a small amount from ethanol 99.5% at 2% (for example 20 mL from ethanol to 1 L LB broth vol/vol). Before adding IPTG, it is very important to monitor OD 600 nm of *E. coli* culture between 0.5 and 0.7. To avoid cell death, amount of culture medium should not exceed the 30% from the container capacity, cover should not be closed tightly, and do not use culture exceeding 0.7 at OD 600 nm of the log phase where *E. coli* growth starting to decrease.

4. To prepare 12% SDS-PAGE (one gel), starting by lower gel preparation, in a 15 mL tube, add 2.55 mL DW, 3 mL 30% Bis-acrylamide (acrylamide 87.6 g, methyl-bis-acrylamide 2.4 g them mess up to 300 mL DW), 1.8 mL 4× lower gel buffer (Tris base 36.49 g added to 100 mL DW pH 8.8, add 0.8 g SDS), 75 μL 10% APS, then just before use add 0.04% TEMED (vol/vol) and mix well. Then, pour it directly to SDS-PAGE tray. To avoid dryness of upper surface of lower gel, cover by an appropriate amount of DW. After solidification of lower gel, prepare upper gel in another tube by mixing 1.75 mL DW, 0.5 mL Bis-acrylamide, 0.75 mL 4× upper gel buffer (Tris base 6.05 g added to 100 mL DW pH 6.8, add 0.4 g SDS, 30 μL APS, and 0.04% TEMED (vol/vol) thoroughly and pour directly to the tray after discarding water and fixing special comb. Usually, lower gel solidify within 30–45 min while upper one from 5 to 10 min. Apply the tray in the chamber with SDS ×1 buffer, (Tris base 60.6 g, Glycine 288.4 g, SDS 20 g, mess up to 2 L DW), and cover the tray by SDS ×1. Remove the comb gently just before placing the samples and LMW marker. In case of optimization of protein expression conditions, testing should include samples from *E. coli* before and after induction of protein expression by IPTG, supernatant and pellet after sonication, eluate and beads after incubation of sonication supernatant with glutathione-sepharose beads. Samples are prepared by mixing 2× loading buffer (0.5 M Tris–HCl pH 6.8 2.5 mL, 10% SDS 4 mL, Glycerol 2 mL, Bromophenol blue 0.1 mL, DW 0.4 mL, DTT 1 g) and exposure to heat shock at 95° C for 5 min. Run

the samples on the SDS gel, staining by Coomassie Brilliant Blue dye for 0.5 to 1 h then destain for 2 h (Destaining solution: Add 300 mL of methanol and 100 mL of glacial acetic acid in 600 mL of DW and store at room temperature).

5. During preparation of thioglycollate medium, wear protective mask and gloves and apply all steps in safety cabinet as possible because it is an immunostimulant substance. Weigh 4.05 g from the medium then mess up until 100 mL DW. After thorough mixing, autoclave the solution where color changed from green to yellow. Let the medium to cool down, and just after the color changed again to green, filtrate the solution rapidly by 0.45 μm filter to avoid hardening of medium, and aliquot in 1.5 mL tube for keeping at – 30 °C until use. You can inject the intraperitoneally by 2 mL and collect peritoneal cells after 3 days of injection.

6. Because our recombinant proteins are expressed in *E. coli*, it is expected to contain variable amount of endotoxin or LPS which is greatly falsified the immunoassays. Endotoxin can be removed from purified protein using commercially available resins. Removal of endotoxin by specific kits is not a warranty that the protein is endotoxin free. Therefore, it must be followed by measuring endotoxin levels against positive control samples as LPS. This process is recommended to perform for every lot of prepared recombinant proteins. Using of polymixin B is important because it indicates that the obtained effect of macrophage IL-12 production is related to protein effect but not to the effect of undetectable levels of resident endotoxin if exist. The cytokine levels against tested protein are importantly to be validated against result of LPS, where significant reduction should be obtained for LPS mixed with polymixin B compared with LPS without polymixin B.

7. Recombinant GST protein is recommended to use as a control in case of protein fused with GST to validate the results because sometimes it has a stimulatory effect against immune response. The rGST is usually easily prepared under many conditions of protein expression. Plasmid without insertion of target gene sequence (e.g., pGEX-4T3 or pGEX-4T1 is transformed in *E. coli* BL21 (DE3). Then, perform all steps as described earlier (Subheadings 3.1.2 or 3.2.2). Finally, check the expression and purity by SDS-PAGE; expected molecular size of GST is 26 kDa.

8. In case of NcGRA6, we additionally used NLA from purified tachyzoite to validate the effects. For preparation, aspirate the old medium from Nc-1 parasites maintained in Vero cells, and then wash once with PBS (10 mL). Aspirate PBS and scrape cells thoroughly. Add 5 mL PBS on flask and suspend the cell

suspension using 27-gauge needle and 5 mL-syringe. Filtrate the suspension using 5 μm filter into 15 mL tube. Do this step at least for three 75T flasks. Centrifuge the tube at 850 × g, 10 min, 20 °C, and then aspirate supernatant from tube. Add PBS (1 mL) to the pellet using pipette and mix well. Transfer new 1.5 mL tube and centrifuge at 5000 × g, 10 min, 4 °C. Aspirate supernatant completely from tube. Add 100 μL PBS to the pellet without mixing. Sonicate tube for 30 s for two times. Then, subject the sample to three cycles from freezing (liquid nitrogen) and thawing (water bath to 37 °C). Depending on turbidly of sample add additional amount 100–300 μL from PBS, mix well by vortex, then centrifuge 10,000 × g, 10 min, at 4 °C. Carefully collect the supernatant and transfer into a clean 1.5 mL tube. Check concentration of lysates using BCA kit and keep at −30 °C until use.

9. For ELISA, it is recommended to use a multichannel pipette and changing the pipette tips after transferring the solution from one well to the next. When working on many samples in multiple plates, it is recommended to add standard in each plate to avoid plate to plate variations. Manual washing is required to avoid blind washing process of automatic ELISA washing machine. The TMB substrate solution should be equilibrated to room temperature before using.

Acknowledgments

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Part VI

Vaccines for Fish

Chapter 9

Fundamentals of Fish Vaccination

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Abstract

Fish health management has become a critical component of disease control and is invaluable for improved harvests and sustainable aquaculture. Vaccination is generally accepted as the most effective prophylactic measure for fish disease prevention, on environmental, social, and economic grounds. Although the historical approach for developing fish vaccines was based on the principle of Louis Pasteur's "isolate, inactivate and inject," but their weak immunogenicity and low efficacies in many cases, have shifted the focus of fish vaccine development from traditional to next-generation technologies. However, before any fish vaccine can be successfully commercialized, several hurdles need to be overcome regarding the production cost, immunogenicity, effectiveness, mode of administration, environmental safety, and associated regulatory concerns. In this context, the chapter summarises the basic aspects of fish vaccination such as type of vaccine, modalities of vaccine delivery, the immunological basis of fish immunization as well as different challenges associated with the development process and future opportunities.

Key words Vaccine, Vaccination, Fish, Aquaculture, Immunology, Correlates, Injection, Oral, Immersion, DNA vaccine, Killed vaccine, Live vaccine, Adjuvant

1 Introduction

The 2030 Agenda for Sustainable Development in fisheries and aquaculture, aims towards eliminating the disparity between developed and developing countries and warranting food security and nutrition to all of humankind without depleting natural resources [1]. To achieve this goal, countries were focusing extensively on their aquaculture potential, as capture fishery production remained relatively static since the late 1980s. As a result, aquaculture has successfully overtaken the capture fishery in the last decade, and becomes the fastest growing animal production sector in the world, contributing about 80 million tonnes of aquatic animals with a value of US$ 232 billion [1]. As a downside of this sector-wide intensification in the production, an increased risk of infectious disease outbreaks is also accompanying the aquaculture industry for the past few decades. It has been estimated that as much as

10% of global aquaculture production is lost due to infectious diseases annually, which amounts to >10 billion US$ [2]. Thus, to sustain the high growth trajectory of aquaculture and for fulfilling the goal of 2030 agenda, the global focus should rely on disease prevention. Vaccination is regarded as the profound tool for prevention and control of fish diseases in terms of economic, environmental and ethical point of view. However, unlike their terrestrial counterpart, fish vaccine development has faced several challenges viz., limited knowledge of the fish immune system, the vast diversity of pathogens and their susceptible host species, difficulties in identification and formulation of antigens, selection of efficient adjuvants and vaccine carriers, challenges related to the mode of delivery, and various laws and restriction related to food fishes. Nevertheless, despite these challenges, fish vaccinologists have made impressive progress over the last four decades, developing 24 licensed fish vaccines which are now commercially available for use in a variety of fish species. These vaccines comprise whole killed, live-attenuated, peptide subunit, DNA and recombinant protein. This chapter summarises the development in the field of fish vaccinology focusing on early progress and current status of fish vaccine development, various challenges associated with it, existing opportunities, and future directions regarding the use of vaccination for control of infectious diseases in commercial fish farming (Fig. 1).

2   Definition and Properties of the Vaccine

The imperial definition of fish vaccine given by Ellis [3] is as follow, "Vaccines *are preparations of antigens derived from pathogenic organisms, rendered non-pathogenic by various means, which will stimulate the immune system in such a way as to increase the resistance to disease from subsequent infection by a pathogen.*" In simple words, vaccines are biologically based preparations containing antigens (a unit of a pathogen or the entire pathogen), intended to establish or improve immunity in the host to a particular disease or group of diseases. It works by exposing the immune system of a healthy animal to an antigen and then allowing the host immune system to develop a response and a "memory" to accelerate this response in subsequent infections by the targeted pathogen. Although, this definition broadly describe the functional mechanism of vaccines, an "ideal fish vaccine" should comprise of the following properties,

1. *Immunogenicity*: The antigen or the foreign substance present in a vaccine should have the potential to induce humoral and/or cell-mediated immune responses in the host.

2. *Immunological memory*: A vaccine will be considered as an ideal one, if it induces long-lasting protective immune memory, i.e., once administered, the vaccine should trigger the host's
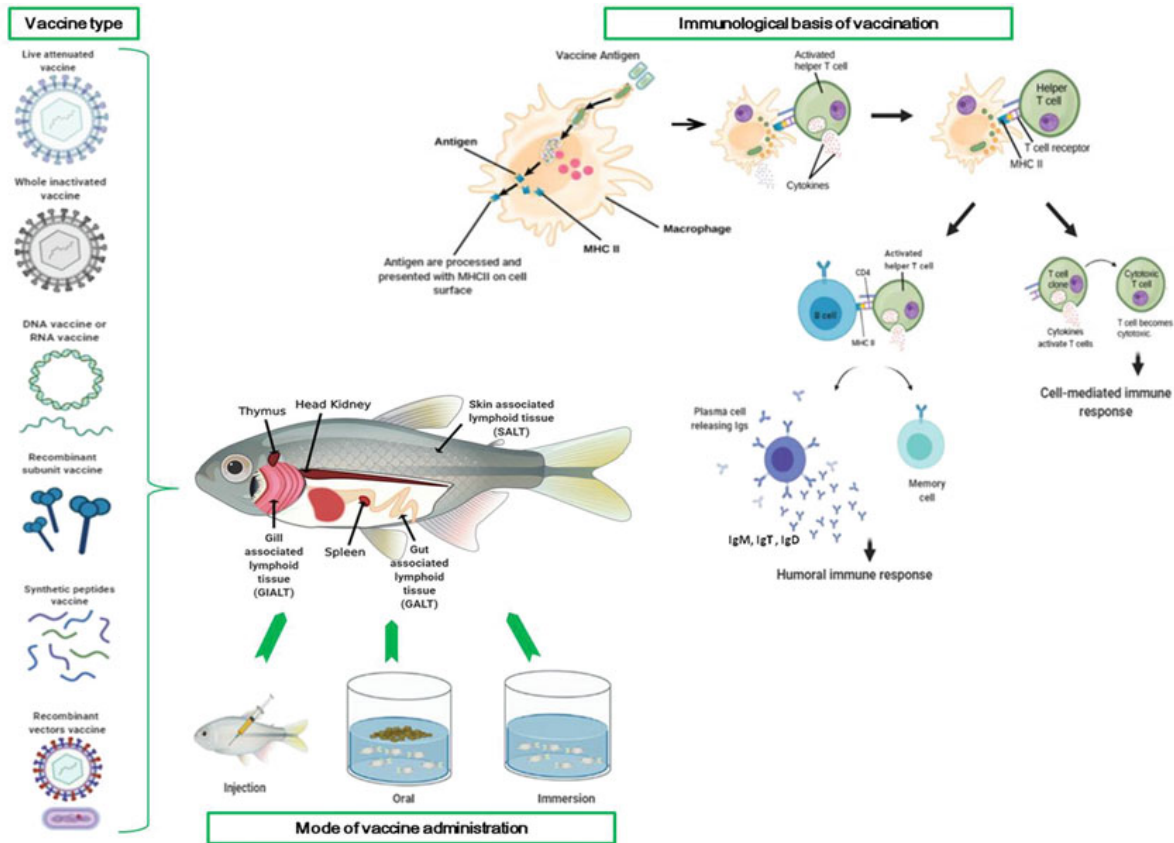
Fig. 1 Fundamentals of fish vaccination—type, mode of administration, and immunological basis of vaccination

memory B cells and helps the host to recognize and combat the antigen immediately on future exposure and provide protection to the fish, at least for a production cycle.

3. *Safety*: In general, a vaccine is regarded as safe if it simulates the natural infection in producing immunity but incapable of producing clinical illness and side effects in the host. In addition to this, the fish vaccine should also be safe for the end consumer i.e., human or other animals, who would ultimately consume the immunised fish.

4. *Broad spectrum of protection*: As individual fish pathogen comprises of wide variety of strains, so an ideal fish vaccine should also give effective protection against the broad spectrum of the pathogen.

5. *Multispecies protection*: Unlike other animal pathogens, which can infect one or two terrestrial species, fish pathogens have a wide range of susceptible host species, so in order to be ideal, the fish vaccine should be equally effective in several fish species.

6. *User-friendly*: User-friendliness is a critical criterion for an ideal fish vaccine. The fish vaccine should be prepared with an aim for non-stressful, time-efficient, and mass immunization protocol.

7. *Cost-efficiency*: Fish vaccine should also address the economic part of the vaccine. It should be inexpensive so that farmers can afford vaccination of their farmed fish and make a profit after selling their produce.

3   Immunological Basis of Fish Vaccine

Fish, despite their low collocation in the vertebrate phylum, possess a complete immune system. Similar to higher vertebrates, the teleost immune system also comprises of a stratified defence strategy with physical barriers to prevent pathogens from entering the organism, an immediate nonspecific response during a breach in the physical barrier, and the adaptive immune system. The adaptive immune responses are activated initially by the innate response such as interferon induction, and later improve its ability of pathogen recognition and retainability of the immune response in the form of immunological memory. Central to the adaptive response are the lymphocytes, the B- and T-cells, responsible for the diversity of antigen recognition, specificity and memory. In fishes, both B- and T-cells are believed to originate from the head kidney, and the maturation of B cells occurs within the head kidney, whereas the thymus is important for the maturation of T-lymphocytes [4–7]. Mucosa-associated lymphoid structures (MALT) like gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT) and inter-branchial gill-associates lymphoid tissue (GIALT) are also integral parts of the fish immune system along with the systemic immune responses [8]. These adaptive immune responses are the basis of formulating "fish vaccine."

The adaptive immune responses are broadly classified into humoral and cell-mediated immune responses. Immunoglobulins mediate the adaptive humoral immunity as in mammals. Immunoglobulins, in addition to their role in systemic immunity, also play an essential role in mucosal immunity. Three classes of immunoglobulin viz., IgM, IgT, and IgD have so far been identified in teleosts of which IgM is the most abundant in the systemic circulation. The IgM isotype shows functional heterogeneity with monomeric, dimeric, and tetrameric forms and are produced by plasma cells and plasmablasts located in the head kidney [9, 10]. There is poor affinity maturation of IgM responses, even though it has been used as a marker for protection against several bacterial and viral diseases [11–13]. The isotype IgT, was characterised in several teleost fish species in multimeric forms and is reported to play a

major role in gut mucosal immunity where its role is similar to IgA of mammals. The third antibody isotype, IgD, a monomer whose secreted form lacks the variable region. IgD has a significant role in skin and gill mucosal immunity. Upon vaccination or being exposed to antigens, this adaptive humoral immune system comes into play in a major way. Sequentially, after vaccination the antigens are processed and presented by antigen-presenting cells (APC) with the help of MHC class II molecule allowing the activation of naïve CD4+ T-helper cells which in turn resulted into cognate interaction between activated T-helper cells and B cells via CD40 ligand. The activated B cells then proliferate, differentiate and secrete specific antibodies (Igs). Secretions of IgM by B cells are maximal in case of intraperitoneal injection vaccination. However, IgM is also reported to be secreted in serum and mucus upon immersion and an oral vaccination. IgT and IgD secretions in MALT are mainly associated with mucosal vaccination where they play important roles in adaptive mucosal immunity.

The other adaptive immune response to vaccination is cell-mediated immunity. T-lymphocytes are responsible for orchestration of the cell-mediated immune responses. In teleost, T-lymphocytes constitute a minor population in circulation, although they are abundant in mucosal tissues. Many of the surface markers used to differentiate between naïve, memory and effector T-cells; however, the functional aspects of putative responses of different T-cell subsets remain unclear. Cytotoxic T-lymphocytes (CTLs) are the effector cells of the cytotoxic responses which express CD8 molecules and TCR co-receptors, important in MHC class-I restriction. Class-I antigens, presented by all nucleated cells, when bound to endogenous foreign antigens such as virus-infected cells, stimulate the CD8+ cytotoxic T-cells for the destruction of the infected cell thus playing an important role in the cell-mediated immunity. Utke et al. [14] reported specific cytotoxicity in VHSV infected cells by peripheral blood from fish immunised with a DNA vaccine encoding VHSV G-protein.

4 Type of Vaccine

A vaccine is classified based on the approach used to develop it. Each approach has its advantages and specific mechanism of action. Based on the feasibility of manufacturing and nature of the infection, the vaccine is designed. The choices of vaccine design are typically based on basic information about the microbe, such as how it infects cells and how the immune system responds to it, as well as practical considerations, such as size and value of fish species in which it is to be administered. Broadly, vaccines can be classified based on antigen delivery systems (Table 1), Replicative Antigen Delivery System—Live-attenuated vaccine, DNA vaccine, Vector

Table 1
Type of vaccines

Vaccine type	Formulation	Immune induction	Advantages	Disadvantages
Replicative antigen delivery system				
Live-attenuated vaccine	These vaccines contain live-attenuated microorganisms which are "weakened" or devoid of disease-causing capacity but still capable of replicating and presenting its immunogenic properties inside the host Methods used for attenuations ranges from chemical/heat attenuation, continuous passaging of the pathogen in different heterologous systems (heterologous animals, tissue culture, embryonated eggs) and genetic attenuation (mutation by deletion, disruption, or insertion of the metabolic pathway or virulence gene)	Elicits both humoral and cell-mediated immune responses	Being self-replicating does not need booster immunisation Provides long-lasting protective immunity to the host Can be administered easily through oral or immersion method	Possess the risk of recombination of different strains resulting in the emergence of the new strain Has the risk of reverting to virulence strain Causes serious threat to off-target animals and the aquatic environment Not suitable for immunocompromised animals as they work on an active immune system
DNA vaccine	DNA vaccination or nucleic acid immunisation involves the delivery of plasmid DNA (raised in microorganisms such as bacteria) encoding a vaccine antigen to the host Under the control of eukaryotic promoters, the plasmid DNA expresses inside	Elicits both humoral and cell-mediated immune responses	Induces strong and long-lasting protective immunity to the host. Possess no risk of inadvertent infection DNA vaccines are stable in dried powder or in a solution and do not need a cold chain [15, 16] The vector can encode the multivalent vaccine for multiple diseases, that could be given in a single administration [17, 18] DNA vaccines are	Legal restrictions (primarily related to genome integration) to the use of DNA vaccine treated food fishes in most of the countries hampers its licensing and commercialisation

(continued)

Table 1
(continued)

Vaccine type	Formulation	Immune induction	Advantages	Disadvantages
	the recipient, first by transcription into mRNA and then by a translation into the protein encoded by the gene The host immune system recognises the expressed antigenic proteins as "vaccine / foreign antigen"		relatively cheap and are easy to produce via identical production processes [19]	
Vector vaccine	Vector vaccines utilize live virus vectors, for transferring antigenic genes into the recipient host which in turn express the encoded protein of another pathogenic microorganism, as the vaccine antigen [20] For developing the fish vaccine, salmonid alphavirus (SAV) replicon vectors are used commonly as these vectors are functional in cells from a wide range of animal classes and express GOI in the temperature range of 4–37 °C [21, 22]	Elicits both humoral and cell-mediated immune responses	Apart from the antigen, the vector has the potential to replicate inside the host cells actively and can activate the immune system like an adjuvant The alphavirus-based replicon has the advantage of the fact that it does not spread/recombinant to other cells after initial replication [22, 23] The particle of alphavirus replicon has a potent ability to improve mucosal immunity [24]	Pre-existing antibodies against the vector virus can neutralize or inhibit the viral vector, thereby reduces the targeted immune responses against the foreign antigen Vector technology is still new for fish vaccine development and has been tested to a minimal extent
RNA vaccine	RNA vaccine consists of an mRNA strand that codes for a virulence factor and/or protective antigen of a pathogenic microorganism On entering inside	Elicits both humoral and cell-mediated immune responses	RNA vaccines are not made from pathogen particles or inactivated pathogen, so are non-infectious Unlike DNA vaccine, RNA vaccine does not integrate itself into the host genome and degraded once the protein is made	Very new technology, so tested to a very limited extent in finfish vaccinology

(continued)

Table 1
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Vaccine type	Formulation	Immune induction	Advantages	Disadvantages
	the recipient, the host cells translated the genetic information of the mRNA strand to produce the antigenic protein This antigen is displayed on the cell surface, where the host immune system recognises it		Limited clinical trial results indicate that these vaccines generate a strong immune response and are well-tolerated by healthy individuals	
Non-replicative antigen delivery system				
Whole-cell inactivated vaccine	These vaccines contain killed microorganisms (virus/bacteria/parasite) that have been inactivated through physical or chemical processes such as heat, formaldehyde or radiation treatment The inactivated pathogens lose their ability to cause disease but remain antigenic or immunogenic to the host, which in turn, recognises the foreign structure of the killed-pathogen, and activates its immune system	The vaccine elicits only humoral immune responses and not a cell-mediated immune response	Unlike live attenuated vaccines, the inactivated vaccine does not carry the risk of mutating back to their disease-causing state Do not require cold chain for storage and can be easily transported in freeze-dried form These vaccines are easy to manufacture and economically feasible	Being inactivated these vaccines induces relatively weaker immune responses, so they need several booster doses for maintaining the adequate level of protective immunity over a longer time To maximize their effectiveness, they require suitable adjuvant Mostly injection mode of delivery is effective
Subunit vaccine	Subunit vaccine uses the recombinant technology where only the immunogenic target regions of a pathogen are used as vaccine antigens and expressed in a heterologous host from which	Elicits both humoral and cell-mediated immune responses	Have no live components, thus no risk of inducing disease Safe, stable, and easy to manufacture	Although very effective against human and animal pathogens, in the case of fish vaccine, the administration of the recombinant antigens found to be inefficient in inducing protective immunity Poor immunogenicity of the antigens [30, 31], induce a less strong immune response

Table 1
(continued)

Vaccine type	Formulation	Immune induction	Advantages	Disadvantages
	the preotective antigen is purified and used in vaccine formulation [20] Biotechnological tools are used for recognition and designing of the gene sequence of pathogen's protective antigen After designing, the antigenic genes are inserted in prokaryotic and eukaryotic productions hosts. They are cultured on a large scale under strictly controlled laboratory conditions by fermentation methodology to produce the antigenic protein The production hosts range from bacteria [25], cell culture [26], yeast [27], insect cells [28], microalgae as well as transgenic plants [29]			Often a response can be elicited, but there is no guarantee that immunological memory will be formed in the correct manner
Toxoid vaccine	Toxoid vaccine is generally developed from exotoxin, secreted by bacteria They are developed by inactivating or reducing the toxicity of the toxin by chemical or heat treatment while maintaining its immunogenicity When the immune system receives a harmless toxoid, antibodies are	Elicits only humoral immune responses	Toxoid has the capacity to trigger an immune response and mount immunological response and memory These are extremely safe method of immunization and are less likely to induce any side effect They can also work in immunocompromised individuals	May require several doses and usually need an adjuvant Relatively low antibody responses are reported from the limited experimental trial of toxoid vaccine in aquaculture, reducing its applicability

Table 1
(continued)

Vaccine type	Formulation	Immune induction	Advantages	Disadvantages
	produced that lock onto and block the toxin, termed as anatoxin			
Peptide vaccine	Peptide vaccine is referred to the complex of synthetic peptides or small amino acid domain and the surface carrier protein, which have the capacity of generating immune responses in the recipient host	Elicits both humoral and cell-mediated immunity	They are very simple and safe	Due to low immunogenicity, their applications are limited in fish vaccinology
Anti-idiotype vaccine	This vaccine comprises antibodies that have three-dimensional immunogenic regions, designated as idiotopes that consist of protein sequences which can bind to cell receptors Idiotopes are aggregated into idiotypes, specific of their target antigen. Thus, anti-idiotypes are antigen mimics that can trigger an immune response in the host	Can elicit both humoral and cell-mediated immune responses	Anti-idiotypes can be purified from serum or can be designed using bioinformatics based molecular docking approach and used as antigen replacement	Yet to be explored in fish vaccination
Edible vaccine	Edible vaccines are plant-based vaccine prepared by molecular farming where whole plants or plant cells/tissues are cultured in vitro for the production of immunogenic proteins [32]	Can elicit both humoral and cell-mediated immune responses	These are potentially cheap to produce and are a viable alternative to mainstream production systems such as microbes and mammalian cells cultivated in large-scale bioreactors Unlike other recombinant technologies, they are free from undesirable	This vaccine technology is at an early stage for fish vaccines [33] but likely to develop in the near future

(continued)

Table 1
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Vaccine type	Formulation	Immune induction	Advantages	Disadvantages
	Edible vaccines, after consumption, expresses the antigenic proteins, which are then transported via specialised M-cells to the dendritic cells subsequently activating a coordinated immune response involving B cells and T-helper cells as the main factors		components, e.g., endotoxins in bacteria, and hyperglycosylated proteins produced by yeast. There is no limit to their production scale, and the cost of scaling up is low	

vaccine, RNA vaccine; Non-Replicative Antigen Delivery System—Whole-cell inactivated vaccine, Subunit vaccine, Toxoid vaccine, Peptide vaccine, Anti-idiotype vaccine, Edible vaccine.

5 Modalities of Administration of the Fish Vaccine

Although, type of vaccines depending on the *nature of the antigen*, i.e., non-replicating or replicating vaccines and *Recombinant technologies* are described above in Table 1, development of fish vaccines depends on other criteria—*Mode of delivery* i.e., vaccination via mucosal surfaces (immersion or oral) or injected. The different modalities of vaccine administration are discussed as follows:

1. *Injection* vaccines: Injection mode of vaccination is the conventional approach of vaccine delivery to fish. The intraperitoneal route is used for delivery of most of the vaccines viz., live-attenuated vaccine, whole-cell killed vaccine, subunit vaccine; whereas, the intra-muscular route is preferred for DNA vaccines [34]. Injection route is also the most potent and gives a high level of long-lasting immunity to the recipient host. The long-term protection of injection vaccine is believed to be dependent on the depot effect (retention of antigens at the injection site) [35]. Although the efficiency of the injection vaccine in pertaining long-lasting immunity in high-valued fish like salmon is well-perceived, its labor intensiveness and

non-feasibility for small-sized fish (<20 g) limited its applicability for other non-salmonid fishes [36]. Though the method is highly stressful, injection vaccines which are often multivalent (containing either different bacterins or a combination of bacterins and killed virus or viral proteins) offer challenges to the host immune system such as antigenic competition, interference between antigens and non-specific immunosuppression [37]. Also, their over-dependency on adjuvants for enhancing immunogenicity has the drawback of formation and persistence of visible injection site lesions like adhesion, melanisation, inflammatory response with local or diffuse peritonitis, invasion of fibroblasts and lymphocytes and multiple granulomata [38–40]. Nevertheless, due to its high efficiency, several injection vaccines for important fish pathogens have been developed over the years.

2. *Immersion* vaccines: Immersion vaccination implies immersion of fish in water containing vaccine antigens, wherein the antigens are taken up by the skin, gills or gut and processed by the immune system, resulting in eliciting a protective immune response. Immersion vaccination involves two methods; dip vaccination (high dose vaccine for a short time) and bath vaccination (diluted vaccine for a more extended period). The immersion (dip or bath) route constitutes the simplest form of vaccine delivery system for fish. It is proved to be a very effective method for mass vaccination of small-sized fish and fry (<0.5 g), when they are considered to be adaptively immunocompetent. However, due to limited uptake of antigens compared to injection, the immersion route resulted in providing short-term immunity or moderate to low protection in most instances, even though many exceptions exist [41]. The lower efficacy of immersion vaccine depends on several variables such as vaccine (antigen) dosage [42], duration of immersion [42], type of vaccine (replicative/non-replicative) [41], nature of antigens (particulate/soluble) [43], fish size (age) [44], osmolarity [45, 46], temperature [47], adjuvant performance [48–52], prime boost strategy [41, 50], mucosal integrity [53, 54], and challenge strategies (virulence and dose of the challenge pathogen, injection/bath challenge). Thus, for developing efficient immersion vaccines, fish vaccinologists are considering various ways to address these variables. As a result, several immersion vaccines have successfully passed the laboratory trials and even gets licensed for commercial marketing.

3. *Oral* vaccines: Oral mode of administration of vaccines to fish seems to the ideal method as it is non-stressful and useful for vaccinating all sizes of fish. But the development of efficacious and safe oral vaccines has been a challenge due to some significant limiting factors. The formulation of oral vaccines with the

feed itself has some challenges such as the vaccine, being incorporated into feed, has to withstand the very high temperature and pressure associated with the feed manufacturing and extrusion process. Besides, the oral vaccine antigens need to be stable in the highly acidic gut (stomach and foregut) environment without degradation before reaching the hindgut wherein they can be processed by the immune cells [55, 56]. In addition, there is also the potential risk of tolerance induction upon oral vaccination, especially in immunologically immature young fish [57, 58]. The other fundamental challenge with oral vaccination is to ensure administration of even dosage throughout the targeted population of fish to be vaccinated. Since fish are fed in large groups with diverse behavioral patterns and feed uptake within each group, it causes difficulty in delivering an adequate amount of vaccines to all of the targeted fish. Also, from an economical standpoint, the volumes of antigen required for this method are much higher than those required for individual injection vaccination. As a result, most of the oral vaccines developed against fish pathogens showed only short-term protective efficacies and failed to confer protection on long duration. Although considerable effort has been dedicated to the development of efficient oral vaccination strategies that can provide stronger and longer-lasting protection in fish, only a small proportion of commercially available fish vaccines are administered orally [34, 59].

In addition to the different modes of vaccine administration, two aspects of fish vaccine need to be mentioned here which are researched extensively for the formulation of successful fish vaccine viz., improvements of adjuvants for injection vaccines and intervention of nano/microparticles for mucosal vaccines.

Adjuvants: *Adjuvants are most important factors for enhancing the immune responses and protection elicited by inactivated vaccines (mostly injectable) against various fish pathogens. Traditionally, adjuvants are described as groups of structurally heterogeneous compounds that are capable of modulating the intrinsic immunogenicity of an antigen [60]; thereby increasing the magnitude of an adaptive response to a vaccine (potency) or ability to prevent infection and death (efficacy) [61]. They are classified according to their immuno-modulating capacities, Signal 1 (presentation of antigen) and Signal 2 facilitators (additional secondary signals) [62]. Although, both the Signal 1 and Signal 2 facilitators act towards the activation of specific T and B lymphocytes [63], their mode of action varies. Signal 1 type adjuvant influence the fate of the vaccine antigen in time, place and concentration thereby improves the immunogenicity of the antigen. Signal 2 type adjuvants provide co-stimulatory signals during antigen recognition phase and also render a conducive environment for antigen-specific immune responses [61].*

Among the Signal 1 adjuvants used in fish vaccinology, Freund's complete adjuvant (FCA) composed of heat-killed mycobacteria and mineral oil with a surfactant, [64] has been the most common. However, its usage has not been successful for fish vaccination as it is associated with severe side effects like injection site granuloma. For this, Freund's incomplete adjuvant (FIA) was developed, which lacks the mycobacterial components of the FCA emulsion. Although, FIA has proved to be highly effective in fish vaccination and have a significantly reduced level of toxicity, still it proves to be not free of unwanted side effects like peritonitis as described by Gjessing et al. [65] in Atlantic cod (*Gadus morhua*). Thus, in order to reduce the post-vaccination intra-abdominal lesions, intensive research is done and many products have come through. These are manufactured and commercialised as "Montanides" by Seppic. Montanides are based on either mineral oil, non-mineral oil or a mixture of both and used to manufacture different type of emulsions, water-in-oil, oil-in-water or water-in-oil-in-water, for use in the fish vaccine [66, 67]. Several studies have shown that Montanide adjuvant is a good candidate for an efficacious vaccine against different fish bacterial pathogens [68–70].

Beside the Signal 1 adjuvants, the new class of Signal 2 adjuvants has been gaining importance for use in fish vaccination. Recent studies revealed that these co-stimulatory adjuvants when delivered alongside vaccine antigens, can act as potent activators of several inflammatory cytokines, acute phase proteins, complement genes and antimicrobial peptides which in turn help the recipient fish to obtain effective protective immunity. Examples of various Signal 2 adjuvants for fish vaccines that showed promising results are as follows, β-glucans [71–77], alums [78–83], saponins [84], poly I:C [85–89], synthetic oligonucleotides [90–94], cytokines [95–100] and flagellin [79, 80, 101, 102].

Nanoparticles/Microparticles: *The use of microparticles or nanoparticles as carriers for vaccine delivery has become an important research area for development of fish mucosal (oral/immersion) vaccine [103]. Formulation of nano/micro vaccines involves covalent linkage or physical entrapment. Based on the physicochemical properties (size, shape, surface charge, and hydrophobicity) of the particles, the association of antigen(s) with nano/microparticles can be of three types—conjugation, encapsulation, and adsorption. Compared to adsorption, where the antigen is non-covalently and physically incorporated in the interior of the nano/microparticle, covalent coupling in conjugation (cross-linking antigen to the surface) and encapsulation techniques offers several advantages viz., the requirement of the lower amount of antigen; more efficient processing and presentation the antigens with the help of the APCs; gives higher stability to the encapsulated or conjugated antigen during storage*

as well as in the hostile gastrointestinal environment; facilitates sustained release of the antigen that helps to induce the immunostimulatory properties of the vaccine [61, 104, 105].

With the advent of the nanotechnology in fish vaccinology, a range of different nanoparticles, both natural and synthetic, are being investigated for efficient incorporation and delivery of vaccine antigen into fish via oral or immersion routes. These particles are broadly categorised as polymeric nanoparticles, inorganic nanoparticles, nanoliposomes, immunostimulating complexes, virus-like particles, and nanoemulsions. Among them, natural polymers like Chitosan (copolymer of β-(1–4)-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose, derived from deacetylation of chitin) and Alginate (copolymer of β-D-mannuronic acid and α-L-guluronic acid found in the cell wall of brown algae), synthetic polymer like poly (d, l-lactide-coglycolic acid) (PLGA), inorganic nanoparticles like Carbon nanotubes (CNTs), and Calcium-phosphate nanoparticles and Immune-stimulating complexes (ISCOMs) are extensively studied for fish vaccine research and are reported to be very useful in formulating successful fish mucosal vaccines.

6   Challenges in Fish Vaccine Development

Although there has been much progress in the field of fish vaccinology, various challenges still impediment the development of the fish vaccine. The most critical problem that limited the vaccine development process is the identification of protective antigens. Identifying protective antigens is not easy and requires a variety of approaches viz., pathogen type, fish species, administration method, antigen production method, and availability of challenge model for testing the efficacy of the vaccine candidates [2].

1. Pathogen type: Piscine pathogens are highly heterogeneous in nature and have a diverse range of antigenic epitopes. Although majority of the successful commercial fish vaccines are killed whole-cell bacteria preparations with intraperitoneal injection modalities, the applicability of this approach seems to be limited for other fish pathogens, especially for intracellular or complex pathogens (e.g., virus and parasites), those are difficult or expensive to culture.

2. Fish species: The diversity of fish species itself poses a challenge in vaccine development. As most of the fish pathogens have a wide range of susceptible host and each fish species behave differently to elucidate host-pathogen interactions. So, there are no universal formulae of developing a vaccine against a pathogen which will be equally effective in each of its susceptible hosts.

3. Administrative methods: Method of vaccine administration is the biggest challenge which needs to be determined. It is noticed that some novel vaccines being developed are protective, but current administration methods and vaccination strategies are not appropriate for optimal efficacy (e.g., may need prime/booster vaccination). Although injection method is commonly used for Atlantic salmon, administration of vaccines via the mucosal route is also more practically viable for lower-valued fish species e.g., tilapia and *Pangasius.* However, various challenges have hampered the development of mucosal vaccines, including lack of correlates of protection, lack of optimization of protective doses required, the possibility of oral tolerance, potential denaturation of oral vaccines in the stomach, and the ability of antigens to cross mucosal barriers to gain access to antigen-presenting cells (APCs) [41].

4. Antigen production method: For developing an effective vaccine, selection of protective antigen is utmost important. In addition to selection, the cost of production of the antigen is also equally important so that the developed vaccines can be affordable to low-middle income fish farmers.

5. Challenge models: Evaluation of vaccines efficacy requires standardised *in vivo* disease challenge models that closely simulate the natural infection route. Bath and cohabitation challenge best fulfil the requirement of natural exposure, but they are more challenging to control and standardize than the injection challenge methods. Also, injection challenge is not an appropriate challenge method to test a mucosal vaccine (e.g., dip immersion) administered to fry. Further, some pathogens are incapable of producing diseases in experimental challenge models unless some scarification or stress is used [106, 107]. Thus, in the absence of experimental disease challenge model, determination of vaccine efficacy is considered to be a problematic area, which needs to be sorted out in future.

7   Opportunities and Advancement for Fish Vaccine Development

Despite the difficulties that hindered the development of the fish vaccine, opportunities also prevail for fish vaccinologists to use novel technologies and vaccination strategies which can help in overcoming the challenges. With the advancement in the field of bioinformatics, the cost of whole-genome sequencing of pathogens has reduced substantially [108], enabling targeted vaccine design for heterogeneous species. For example, Ngo et al. [109] has recently characterised more than 300 *Flavobacterium psychrophilum* species from UK and developed an effective trivalent whole-cell vaccine, wherein unwanted immunosuppressive epitopes are

eliminated, and only specific protective antigens are included thereby enhances the efficacy of the vaccine. Likewise, in reverse vaccinology, latest software programmes are being used nowadays to identify highly immunogenic vaccine candidates for the development of protein subunit vaccines or DNA vaccines. After long-pending legalization procedure and extensive safety studies, DNA vaccines are now authorised for use in Europe [110], thus creating a huge opportunity for their growth in the future. Other alternatives to DNA vaccines, such as mRNA vaccination or edible vaccine technologies which have proven to be highly efficient and safe for other animals can be used for prophylactic and therapeutic applications in fish also. In case of live-attenuated vaccines, traditional attenuation methods are being replaced by molecular methods wherein, genetically modified pathogens can be produced which permit better control and safety than random mutations in live vaccines [111], however, their classification as GMOs has restricted their use in aquaculture, but with further safety measures may be permitted in the future.

Vaccine administration strategies also provide huge scope for future researches. Different nanomaterials (<1000 nm) such as virus-like particles (VLPs), liposomes, ISCOMs, polymeric, and non-degradable nanospheres showed potential as antigen delivery vehicles, allowing the sustained release of antigens and also acts as immune enhancer adjuvants [112–114]. These delivery systems are being experimentally used for mucosal delivery of fish vaccines and considered to be the immediate focus area for development in fish vaccinology. Furthermore, regarding vaccination strategies, prime-booster vaccination with early immersion vaccination then IP booster vaccination, or IP booster vaccination followed by oral booster vaccination, has reported helping in the stimulation of both mucosal and systemic immunity (an important criterion for fish vaccine development), thereby, generate opportunities for future researches in this direction.

8   Correlates of Vaccine Protection Efficacy

Like the current opportunities in the area of vaccine development and administration strategies, establishments of *in vitro* correlates for vaccine protection efficacy also opens up new avenues for future research. The current *in vivo* challenge methods use a large number of fish in vaccine testing, which is considered to be unethical and economically non-viable. Alternative non-lethal, quantitative immunological methods are required, which can determine the protective efficacy of the developed vaccine without sacrificing the immunised fish. Also, the prevailing challenge models seem to be unreliable for various pathogens, where there is inconsistent mortality or no mortality at all like infection with (PRV) [115] or

PMCV [116]. For this, 3 different correlates of vaccine protection viz., antibody titer, expression analysis of surrogate markers of protective immunity and antigen dose, can become important benchmarks in optimising vaccine production in aquaculture [117].

Antibody Titer: *It is the most commonly used correlates of protection for the licensure of vaccines in mammals; however, in fish; this method is yet to be established as a signature of protective immunity. Passive immunization can help in evaluating the protective effect of antibodies [118]. Various studies have shown positive correlation between antibody titers and level of post challenge protection in passively vaccinated fishes, e.g., in Nile tilapia (*Oreochromis niloticus*) against* Streptococcus agalactiae *[119],* Streptococcus iniae *[120]; in Rainbow trout (*Oncorhynchus mykiss*) against* A. hydrophila *[121],* Flavobacterium psychrophilum *[122],* Streptococcus iniae *[123], Infectious hematopoietic necrosis virus [124],* Yersinia ruckeri *[125], Viral hemorrhagic septicemia virus [126],* Vibrio anguillarum *[127]; in Indian major carp (*Labeo rohita*) against* A. hydrophila *[128]; in Coho salmon (*Oncorhynchus kisutch*) against* Aeromonas salmonicida *[5, 6]; in Channel catfish (*Ictalurus punctatus*) against* Flavobacterium columnare *[129],* Streptococcus ictaluri *[130],* Ichthyophthirius multifiliis *[131], in Atlantic salmon (*Salmo salar*) against* Aeromonas salmonicida *[132], Infectious pancreatic necrosis virus [11, 114]. Although these studies give a comparative account for antibody as "correlate of protection" but still there are problems in confirming the protective threshold (for a vaccine) which can confer protection in vaccinated individuals. The main problem is the differential behavioral pattern of individuals in the same group of vaccinated fish; as a result, there is disparity in their antibody titer values which in turn hinders the correlation method. The other problem is the lack of immunoassays to quantify IgT levels in/on mucosal surfaces in response to vaccination. Fish showed well defined compartmentalization of immunoglobulin (Ig) isotypes distribution [133] with IgM provides systemic protection and IgT attributed with mucosal immunity. So, appropriate diagnostic tools for measuring IgT titers expressed on mucosal surfaces is needed to correlate further the antibody response with vaccine efficacy, particularly mucosal vaccine.*

Expression Analysis of Surrogate Markers of Protective Immunity: *"*Surrogate markers*"* of protective immunity are genes which can be used as bio-signature of vaccine protection for antigens that evoke immune responses. Vaccine antigens are usually presented through MHC-II molecules to T-cells, while DNA vaccination is mediated through the MHC-I route; however, it is important to note that, both responses can be triggered simultaneously with one prevailing, as the immune response develops [118]. Surrogate markers are important indicator of protective immunity for those

vaccines, where cellular immune responses constitute a crucial function in addition to antibody stimulation. Although, various studies related to DNA vaccine and attenuated live vaccine (against intracellular pathogens) reported upregulation of $CD4^+$ and $CD8^+$ T-cell responses together with Th1 cytokines IFN-γ and Mx [14, 134–139], none of them could show a direct correlation between the quantity of activated (by vaccination) CD8+ T-cells or Th1 cytokines with vaccine efficacy such as RPS, reduction in the number of infectious agents and prevention of pathology in target organs after challenge. Nevertheless, these studies ascertained that identification of surrogate markers for the fish vaccine could be a novel area of future research which can replace lethal challenge study for vaccine efficacy determination.

Antigen Dose: *Antigen dose in vaccine preparations can also act as correlate for vaccine protection in fish. Various studies have reported that a difference of log_{10} in antigen dose directly affects the protection incurred by vaccines. For example, Yamashita et al. [140] showed that, inactivated whole-cell vaccine with antigen dose $>10^{7.5}$ showed higher protection in red-spotted grouper against nervous necrosis virus (RGNNV) challenge; Munang'andu et al. [11] reported that inactivated whole-cell vaccine with antigen dose of 2×10^{10} $TCID_{50}/mL$ and 2×10^9 $TCID_{50}/mL$ corresponded with PCSP>90% and < 42% respectively, in Atlantic salmon vaccinated against IPN; Li et al. [141] and Huang et al. [142] showed an antigen dose dependent increase in protection levels in tilapia vaccinated against S. agalactiae in their separate findings. Likewise, for recombinant protein and DNA vaccines, antigen dose is reported to be a crucial factor determining the protective efficacy, e.g., in rohu against A. hydrophila [143]; in striped bass against Mycobacterium marinum [144]; in Japanese flounder against Vibrio anguillarum [145]; in European Sea Bass against Betanodavirus [146]; in Atlantic halibut against Nodavirus [147, 148]. Overall, these studies show that a cutoff limit of antigen dose can be established that correlates with protection to serve as benchmarks, against which all future vaccine batches can be assessed, i.e., when antigens are identical, vaccines with antigen dose at or above the protective antigen dose can be considered as protective while vaccines below the protective antigen dose would be considered as suboptimal.*

9 Conclusion

In conclusion, it can be said that fish vaccine development has achieved many milestones over the last four to five decades, but it still needs to go a long way. With newer knowledge regarding fish immunology and host-pathogen interaction as well as the advent of novel vaccine technologies from human and veterinary sciences,

fish vaccinology has huge prospect to excel especially in the area of next-generation vaccines. As already mentioned, the success of fish vaccinology relies on the development of non-stressful multicomponent, cost-effective mucosal vaccines, and thus it should get much attention presently. In addition, there is also a growing need for determination of correlates for vaccine protection which is lacking in the fish vaccine, demands proper focus in future researches. Lastly, with more and more emerging diseases, fish vaccine development should accelerate with rapid pace to cope up with the huge demand for the effective vaccines to reduce the damaging effects of diseases in the aqua sector and meet the goal of "2030 agenda" of sustainable aquaculture.

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Teleost Fish as an Experimental Model for Vaccine Development

Marco Antonio de Andrade Belo and Ives Charlie-Silva

Abstract

Advances in vaccine development depend on animal models to test innovative therapies. Recent studies have reported the successful introduction of teleost fish as a new vertebrate model in scientific research, with emphasis on the species *Danio rerio* (zebrafish). This chapter aims to give an overview of important aspects related to the immune system of fish, as well as the current progress of the successful use of these animals in studies for the development of vaccines, assisting in the determination of efficacy and clinical safety. Among the advantages of using fish for the development of vaccines and immunomodulatory drugs, it is worth highlighting the reproductive capacity of these animals resulting in a high number of individuals belonging to the same spawning, transparent embryos, low cost of breeding and high genetic similarity that favor translational responses to vertebrate organisms like humans.

Key words Zebrafish, Fish immune system, Innate or adaptive immunity, Vaccine design, Vaccination

1 Introduction

From an evolutionary point of view, bony fish are considered an excellent model and an indispensable component of comparative immunology [1]. The basal position of fish in the phylogeny of vertebrates makes them very attractive, as the immune system of the fish has a significant functional similarity to that of higher vertebrates, although these aquatic organisms have a free life since the embryonic stages, depending mainly on their innate immune system to survive [2]. The host's responses to invading pathogens are basic physiological reactions mediating a series of defense mechanisms to ensure cell integrity, homeostasis, and survival [3].

The emergence of the zebrafish (*Danio rerio*) as a new model organism and the advancement in genome sequencing and bioinformatics technology has greatly accelerated the discovery and functional delineation of genes associated with immunity in fish [4], substantially increasing the knowledge of the immune response
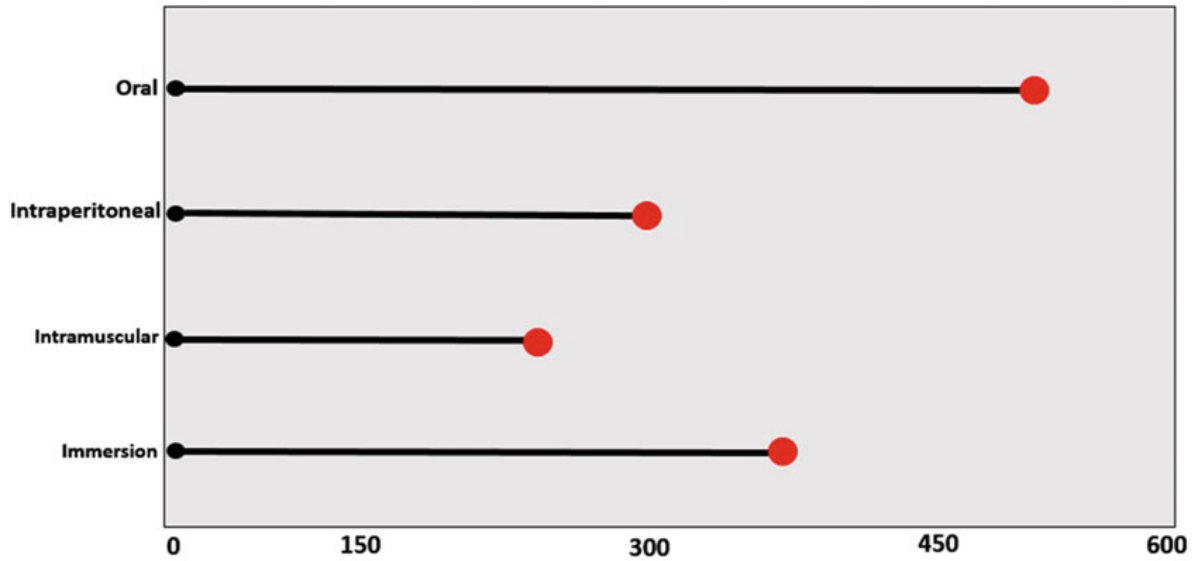
Fig. 1 Fish immunization method ($N = 1350$ articles, database "fish model" and "vaccine ")

pathways, favoring their use in vaccine development. Based on the advantages of fish as an experimental model, the number of articles published has increased considerably in the past two decades. To evaluate this growth, we conducted a careful search in the main databases (such as PubMed, Scopus and Web of Science using a Bibliometrix R Package software) from 2000 to 2021 using the terms "fish model" and "Vaccine" in the subject area. The total number of articles published were on PubMed ("fish vaccine" = 3079 results and "fish model" and "vaccine" 1350 results). Among these vaccine studies, we found that oral vaccination was the most studied method, followed by immersion (Fig. 1). Both are considered natural routes of administration. Secondly, studies involving parenteral methods by intraperitoneal and intramuscular administration.

We also conducted a more specific search on these databases, including the words "Vaccine" and "Zebrafish." This generated 503 articles up to April 2021, and the first research was published in 2000. United States followed by China, Italy, Spain, and South Korea were the countries with the greatest scientific contribution in this area (Fig. 2). The top five journals in terms of number of articles published were Fish & Shellfish Immunology (82), Vaccine (48), Developmental and Comparative Immunology (25), Plos One (18) Journal of Fish Diseases (17), Diseases of Aquatic Organisms (13), and Frontiers in Immunology (12).

In this context, immunorelevant genes associated with the innate and adaptive immunity of fish, including those encoding cytokines, complement system, lectins, immunoglobulins, and cell receptor molecules are being characterized [4]. Extensive studies
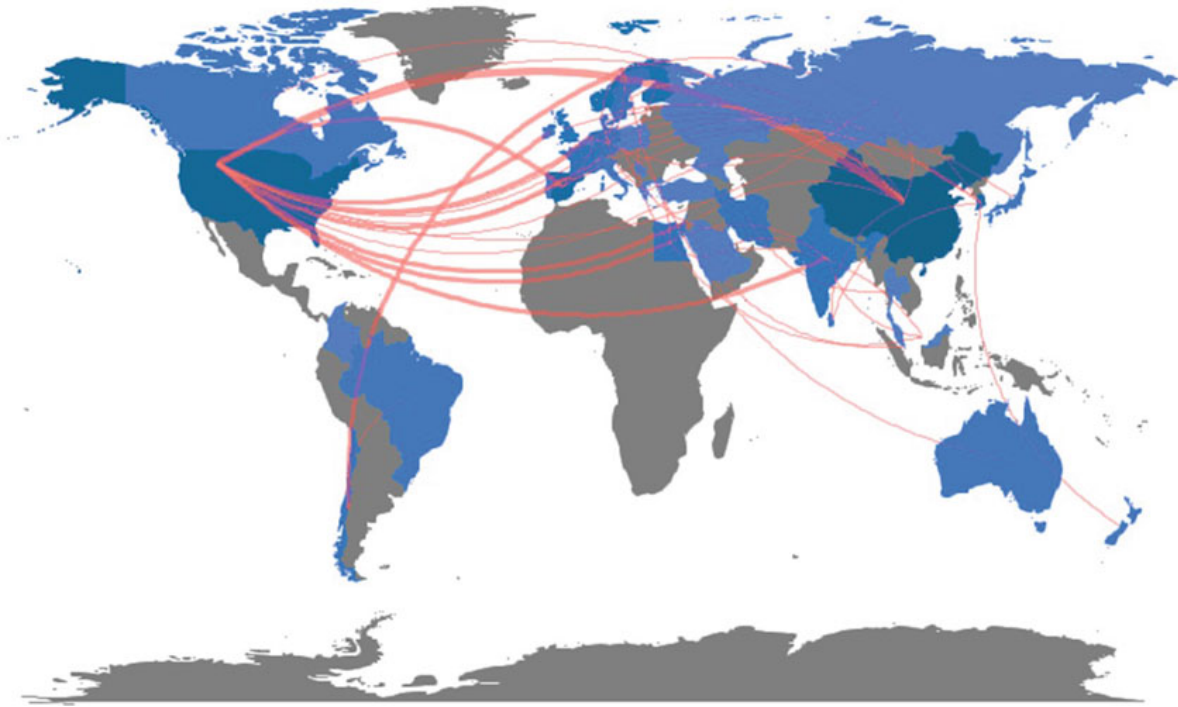
Fig. 2 Country collaboration network ($N = 503$ articles) displays another example of a bibliographic network considering collaboration links between countries. A scientific collaboration network is a network where nodes are authors and links are co-authorships

on the immune system of fish show that there are many molecules and mechanisms that share homology with humans [5]. The phylogeny and evolution of the immune system help to understand the various strategies for disease resistance used by these organisms. Another relevant point in the use of fish in studies of comparative immunology, refers to the modulation of defense responses associated with external and internal factors, considering that the immunity of these organisms can vary with environmental changes, stress, stocking density, among others. These factors can result in suppressive effects on defense responses similar to those observed in mammals, allowing its alternative use for prospecting and evaluation of substances with immunomodulatory potential [6].

Among the advantages of using fish for the development of vaccines and immunomodulatory drugs, it is worth highlighting the reproductive capacity of these animals resulting in a high number of individuals belonging to the same spawning (minimizing experimental statistical errors), transparent embryos, low cost of breeding and high genetic similarity that favor translational responses to vertebrate organisms like humans.

2   Fish Immune System

The ontogeny of the fish immune system is highly complex, mainly due to the great variation that exists between the species [7, 8], the information being restricted to some species of bony fish, among which the zebrafish stands out. However basic questions including the identification of the first lympho-hematopoietic sites, the origin of T and B lymphocytes and the acquisition of complete immune capability are still not fully elucidated [9]. According to these authors, the first hematopoietic site of the zebrafish is an intraembryonic locus, with erythrocytes and macrophages being the first blood cells to be identified in embryos. In zebrafish, myelopoiesis begins inside the embryo in the anterior lateral mesoderm [10].

The thymus, head kidney and spleen are the main lymphoid organs of bony fish. A comparative account of the immune organs between human and fish has been shown in Fig. 3. The thymus participates in the maturation of functional T cells during adaptive immune responses. The thymus is the first organ to become lymphoid, although earlier the kidney may contain hematopoietic precursors, but not lymphocytes [9]. The zebrafish thymus grows strongly until puberty and undergoes involution when the fish reach reproductive age [11]. The dominant hematopoietic organ of most teleost fish is the head kidney, presenting hematopoietic, immunological and endocrine functions [12], presenting an analogy to the bone marrow of higher vertebrates which functions as the primary hematopoietic tissue and lymphoid organ. During the larval phase, the spleen is more erythropoietic than lymphopoietic.
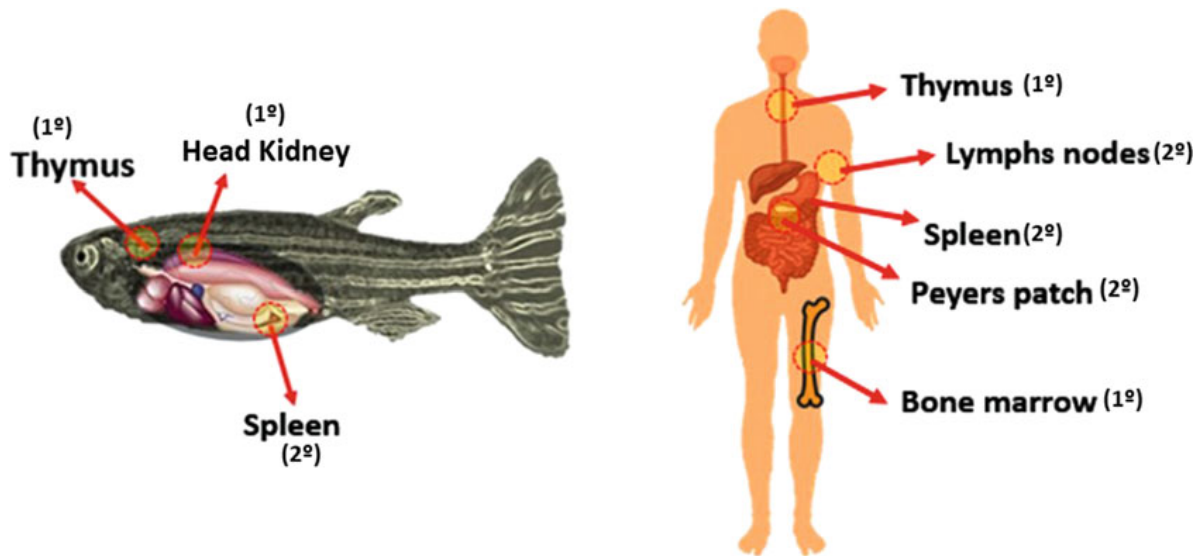
Fig. 3 Comparison of primary and secondary immune organs between humans and fish

Modulated by hematopoietic growth factors, hematopoiesis in teleost fish has the participation of erythropoietin in the production of erythrocytes, G-CSF and GM-CSF in the growth of granulocytes and monocytes, thrombopoietin assists in the formation of thrombocytes, as well as the proliferation and maturation of lymphocytes are mediated by interleukins 1–6 [7].

2.1 Innate Immunity	Innate immunity is part of a complex physiological process that acts by perceiving, integrating, and responding to multiple stimuli, such as infections and injuries, to reestablish homeostasis of tissues, organs, and the organism as a whole [13]. The innate system defends the host from infection by other organisms in a non-specific way, recognizing and responding to pathogens in a generic way [14]. The innate response is the basis of the immune system for the defense of invertebrates and lower vertebrates, being of vital importance for fish resistance to diseases, especially in view of the generally slow acquired immune response and late ontogeny [15].

Characterized in many species, including humans, rodents and teleost fish, pattern recognition receptors (PRRs) are essential for the initiation of innate immune responses, detecting the conserved molecular structure of a pathogen, known as pathogen-associated molecular patterns (PAMPs), inducing the host's immune response [4], and several classes of PRRs have been described, such as Toll-like receptors (TLRs), RIG-I like receptors (RLRs), NOD-like receptors (NLRs) and type C lectin receptors (CLRs). According to these authors, at least 10 and 12 functional TLRs have been characterized in human and mouse, respectively. On the other hand, 17 TLRs were characterized in fish with distinct features and greater diversity when compared to mammals, among which TLR14, TLR19, TLR20, TLR21, and TLR22.

For Magnadóttir [15], the components of the innate immune system are commonly divided into physical parameters, cellular and humoral factors. The first barriers against infection are represented by scales, skin and mucous tissue that produces mucus containing immunological parameters such as lectins, pentraxins, lysozyme, complement proteins, antibacterial peptides, and immunoglobulins. Cellular components such as monocytes/macrophages, neutrophils (granulocytes), non-specific cytotoxic, dendritic, and epithelial cells participate in the innate immunity. Macrophages can secrete IL-1, IL-6, and TNF-a, all of which are indispensable for recruiting macrophages, neutrophils and lymphocytes [16], and cytokines released by phagocytes in tissues can also induce complement and acute phase proteins [5]. All these humoral responses have been found in bony fish and their functions and signaling is being explored with great progress (Fig. 4).

As in mammals, the inflammatory reaction in teleost fish plays a fundamental role in the defense of the organism, which results in
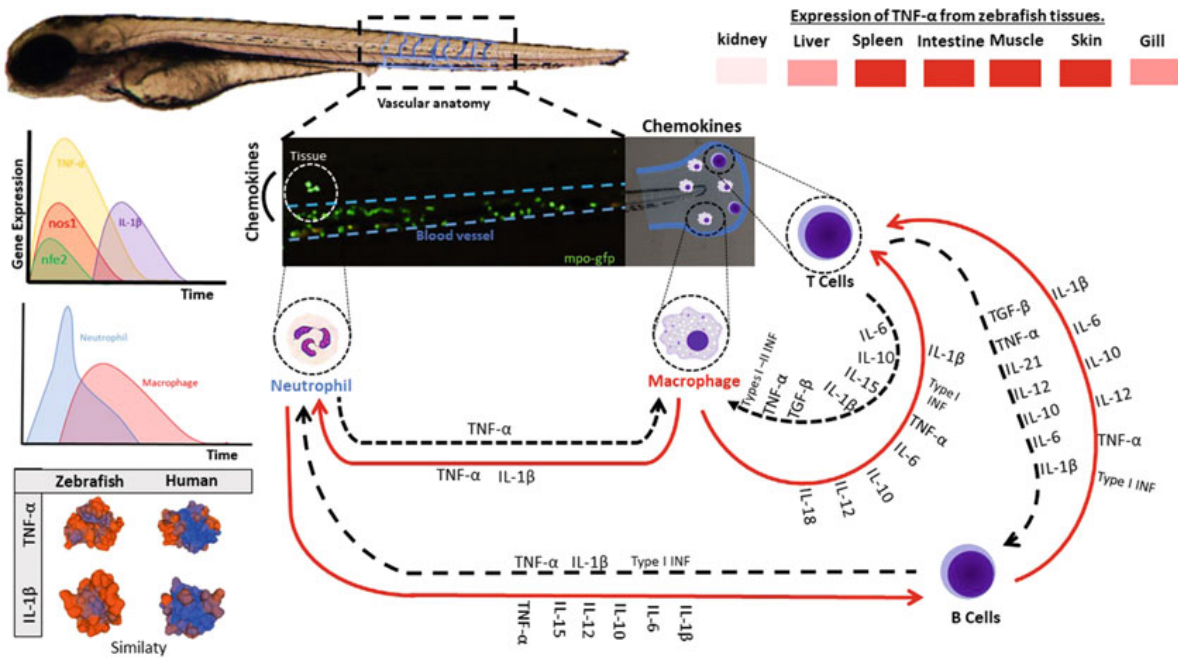
Fig. 4 Schematic representation of cytokines that regulate inflammatory functions in zebrafish

local vascular alterations with increased permeability, edema, increased blood viscosity, leukocyte marginalization, diapedesis, chemotaxis, accumulation of inflammatory cells and phagocytosis [17]. Classic experimental models used in rodents have been successfully adapted to fish to assist in the study of the kinetics of pathophysiological events involving humoral and cellular components during the evolution of acute inflammatory reactions [18–20], foreign body type inflammations [21] and granulomatous inflammations [22, 23]. Therefore, studies of communication between innate and acquired immune systems have received increasing attention in immunological studies in fish.

2.2 Adaptative Immunity

As in mammals, fish show a well-maintained adaptive immune system composed of B lymphocytes which develop from the thymus and head kidney, respectively. The progenitor cells migrate to the areas of the thymus and kidneys, originating from hematopoietic stem cells responsible for the B lymphocyte lineages [24]. In order to study and characterize B cells, these authors developed a transgenic Tg lineage to identify and track the behavior of B cells in zebrafish and reported their appearance only after 20 to 21 h post fertilization. According to the study of in situ hybridization realized by Danilova and Steiner [25], B cells are also produced by the pancreas after 4–11 days of fertilization. The adaptive system in zebrafish is morphologically and functionally mature 4–6 weeks after fertilization [24].

B cells present in many teleost fish have two functions: phagocytosis and production of immunoglobulins. Li et al. [26] reported that several species of bony fish are capable of phagocyting microorganisms, indicating a previously unknown function for these cells in the immunity of primitive animals, although these functions are performed mainly by phagocytes such as macrophages. These authors observed that the B cells of teleost fish have particle absorption activities and induce the activation of phagolysosome formation pathways and intracellular death of ingested microbes. As far as we know, zebrafish B cells do not have this phagocytic capacity either in vivo or in vitro as shown in the study by Page et al. [24]. Our research group has shown that both zebrafish and tilapia produce high titers of antibodies against the new coronavirus (COVID-19). However, these antibodies did not have a seroneutralization effect on the virus, and they only demonstrated a high immunological value for the identification of this microorganism.

The second function of fish B cells is to produce immunoglobulins. The types of immunoglobulins produced by B cells in zebrafish are more limited than those produced in mammals. Unlike immunoglobulins found in mice and humans with immunoglobulin heavy chain isotopes (IgD, IgA, IgG, IgE), only two classes of heavy chain immunoglobulins M and D are present in zebrafish [27]. The zebrafish IgM is an ortholog for human IgM. In Fig. 5, we compare the similarity between zebrafish and humans. However, a new IgZ isotype immunoglobulin (Z) was identified in zebrafish [28].

Zimmerman et al. [29] studied the expression of IgM, IgD, and IgZ genes at all stages of zebrafish development and reported that IgM expression was higher than the other genes in all stages. Several studies have reported that the embryonic phase does not show IgZ expression, and it was only detected approximately 3 weeks after fertilization [24].

The immunoglobulins IgZ and IgT were named differently because they were initially discovered in zebrafish and trout, respectively. However, they share similar characteristics. For example, the IgZ and IgT heavy chains are both composed by four C regions and are highly homologous, indicating that both represent the same immunoglobulin isotype [28, 30], indicating which molecules similar to IgZ-like are a class of immunoglobulins universally found in fish. In addition, other IgZ-type immunoglobulins were characterized in zebrafish as IgZ-like molecule (IgZ-2), a new homologue of the IgZ family, therefore the second IgZ molecule discovered in zebrafish, suggesting the diversity of IgZ family members in the teleost fish [31]. These new IgZ-like class added to the immunoglobulin repertoire raises questions about the evolution of immunoglobulins among mammals and indicates that immunoglobulins in teleost fish are more complex than we previously thought [28]. An interesting fact showing in the study of Hu et al. [31], it
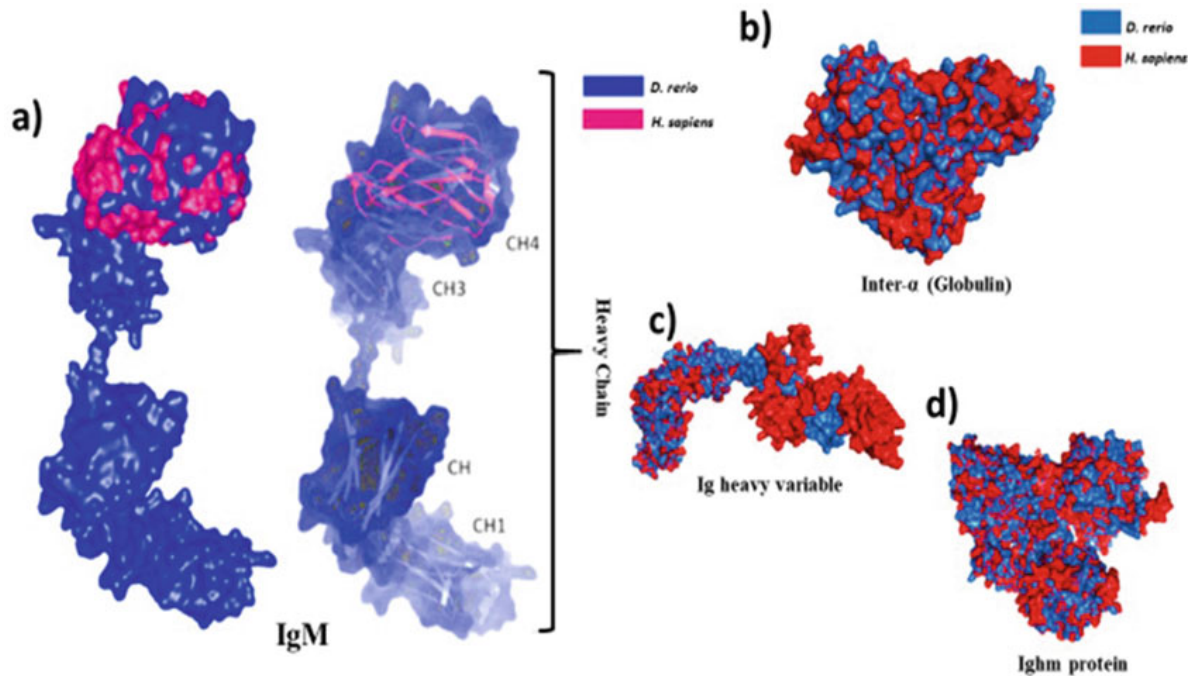
Fig. 5 Comparison of IgMs between human and zebrafish, in blue *D. rerio* and in red *H. sapiens.* (**a**) 3D reconstruction similarity of zebrafish and human IgM heavy chain (**b**) 3D reconstruction and similarity of zebrafish and human globulin heavy chain. (**c**) 3D reconstruction and similarity of zebrafish and human Ig have variable. (**d**) 3D reconstruction and similarity of zebrafish and human IgM protein

was the expression of IgZ-2 in most tissues, and the main insight was the regulation of IgZ-2 expression by LPS. Recently, other members of the IgZ-like subclass such as IgZ, IgZ2, Igτ1, Igτ2, and Igτ3, have been identified zebrafish [32].

2.3 Maternal Immunity

The transfer of maternal immunity is the ability that the mother has in transferring molecules from her immune system to offspring. In mammals, it occurs through the milk and placenta. In fish, this transfer is a little different; fish depends exclusively on the maternal supply of relevant immunity for protection against invading pathogens before the immune system maturation [33]. The adaptive fish system is not fully competent, and there is a window of about 28 days. The zebrafish embryos are released and fertilized externally, and they are frequently exposed in the aquatic environment which is full of pathogens. Previous studies on several species of fish have shown that maternal IgM can be transferred from the mother to offspring similar to mammals [34, 35]. The scheme in Fig. 6 represents the transfer of maternal immunity to zebrafish larvae.

Wang et al. [33] immunized zebrafish females with TNP-BSA, and found that specific antibodies against TNP-BSA were detected in the eggs, demonstrating the ability to passively transfer immunity. This study highlights the transfer of antibodies that can protect embryos in early development against pathogens. In addition,
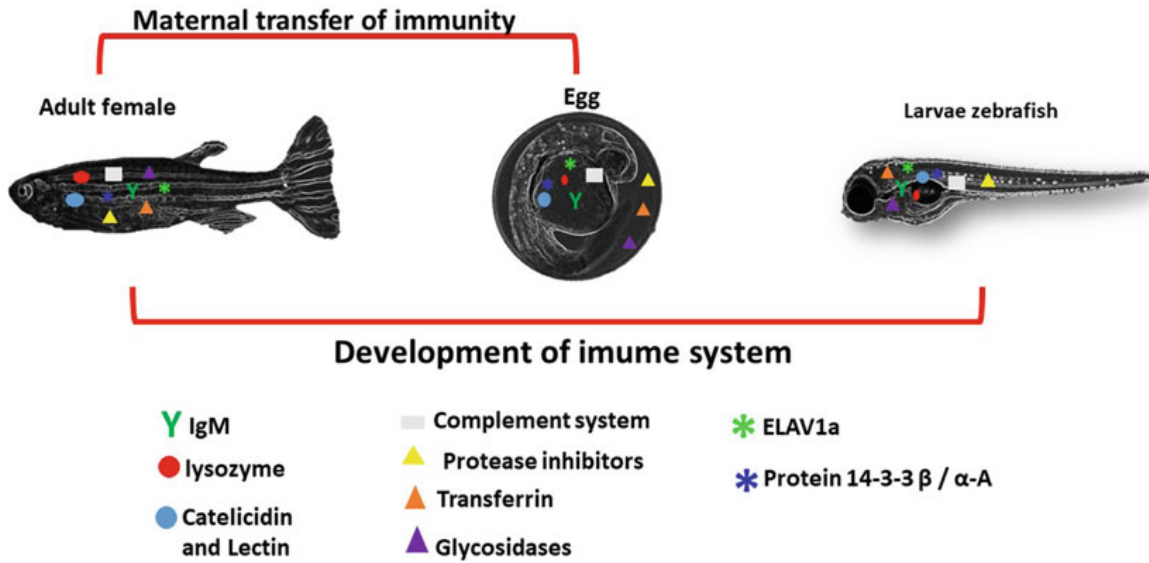
Fig. 6 Schematic representation of the transfer of maternal immunity to zebrafish larvae

other molecules are transferred by maternal immunity: lysozyme, lectin, catelicidin [36]. The maternal transfer of complement system components and their protective role in zebrafish has also been investigated. Embryos derived from the immunized mother are significantly more tolerant to the challenge of *A. hydrophila* than those from non-immunized fish, and the blockade of the activities of the complement system C3 in embryos makes them more susceptible to *A. hydrophila* infection [37].

A study performed by Wang and Zhang [38] reported maternal lysozyme transfer, although this component had been described by other studies, these authors demonstrated its bacteriolytic mechanism, in which maternal lysozyme contributes to the antibacterial activity of egg cytosol. In addition, maternal lysozyme in the cytosol plays a key role in the bacteriological activity of zebrafish eggs, which can be significantly stimulated by cooperation with the complement system.

Recent advances on passive immunity have been made with the aim of identifying substances present in zebrafish eggs through a proteomic analysis of the liquid that covers the embryos called the perivitelline fluid (PVF). Among which were found lectins, protease inhibitors, transferrin, and glycosidases from the beginning of embryogenesis until hatching [39]. These authors carried out in vitro and in vivo experiments with this fluid and demonstrated that PVF had a strong ability to agglutinate bacterial cells and to protect embryos when challenged with the pathogenic bacterium *Edwardsiella tarda*. Another component investigated in embryo protection is the maternal immune factor ELAVL1a that can protect zebrafish embryos from bacterial infection. ELAVL1a is a maternal immunocompetent factor that recognizes LTA and LPS,

as well as Gram-positive and Gram-negative bacteria, killing them through interaction and disrupting their plasma membranes [40]. Wang et al. [41] identified the maternal transfer of a new substance with biological activity against bacteria, the 14-3-3 β/α-A protein, this molecule binds to peptidoglycan that protects zebrafish embryos against bacterial infections.

2.4 Mucosal Immunity

Mucosal surfaces are the main pathways for pathogens to enter all living organisms, being in constant contact with the environment. The mucous membranes are populated by commensal non-pathogenic bacteria, establishing a balance controlled by the mucosal immune system, which helps in the homeostasis of these tissues [42]. Immunomodulation is a prophylactic strategy in bony fish, and the probiotics have been presented this beneficial characteristic with effects on systemic immunity, as well as on mucosal immunity and its influences on the intestine [43]. According to these authors, new perspectives are emerging in probiotic research, for example, probiogenomics to expand knowledge of the probiotics' immunodulatory properties.

Mucosal immunization efficiently induces an immune response of the local mucosa, which may induce the production of local immunoglobulins in MALT, and vaccine stimuli could result in the neutralization of pathogens and prevent infection [44]. Vaccine or infectious stimuli reveal that adaptive immune responses occur on different surfaces of the mucosa of teleost fish by acting on mucosa-associated lymphoid tissues (MALTs), such as: the gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT) and the recently discovered nasopharynx-associated lymphoid tissue (NALT) [45]. For this author, MALT includes diffuse B cells and T cells to defend the mucous environment, responding to mucosal infection or vaccination. Specific antibody responses can be measured in the gills, intestine and mucous secretions of the skin after mucosal infection or vaccination.

B cells and immunoglobulins in MALTs play important roles in local adaptive immune responses of the mucosa, mainly exerted by three immunoglobulin isotypes (IgM, IgD, and IgT/Z) [44]. Similar to mammalian IgA, IgT represents the most specialized class in mucosal immunity and plays indispensable roles in eliminating mucosal pathogens and maintaining fish microbiota homeostasis [46]. During pathogenic infections, specific IgT can be produced locally in mucous secretions, and mucosal sensitivity increases in fish with depletion of this immunoglobulin [44]. For Somamoto and Nakanishi [47], mucosal delivery of fish vaccines administered directly to the mucosa may be used to investigate roles of circulatory and resident T cells in both mucosal lymphoid cells and non-lymphoid tissues.

3   Fish as a Model for Vaccine Development

The development of new immunizers depends on animal models, among them the most used are mice, guinea pigs, non-human primates, rabbit, cattle, dogs, and recently the teleost fish called zebrafish [48]. All current animal models have limitations, for example in a model for the development of tuberculosis vaccines, rodents cannot fully reflect human TB, as they do not form caseous granulomas or develop spontaneous latency. On the other hand, non-human primates reproduce this human disease very well, but their use raise serious ethical questions, as well as taking time to grow and requiring a large number of animals in experimental designs. Generally, mammalian models are relatively expensive and laborious.

One of the great advantages of the Zebrafish model, concerns its daily maintenance cost. Due to its small size (adult measures 3–5 cm), in the same space in which five mice are kept, 60 specimens of Zebrafish (five adult animals/liter of water) can be reared. Resources with infrastructure, labor, and inputs, such as feed and shavings are drastically reduced. It is estimated that for daily maintenance of a mouse specimen is about 13 times greater than a Zebrafish specimen, demonstrating a significant advantage over other models for studying the clinical safety and screening tests of vaccines. Oksanen et al. [49] demonstrated that *Mycobacterium marinum*, a close relative of *Mycobacterium tuberculosis*, causes an infection similar to human tuberculosis in adults of zebrafish thus making zebrafish as a model to study the safety and preclinical efficacy of a new DNA vaccine with antigens (Ag85B, CFP-10, and ESAT-6).

A study by our group investigated the advantages of zebrafish over other models to test the safety of a new vaccine candidate for COVID-19 [50]. In this study, the zebrafish different from the mouse models showed better immune responses, because the zebrafish shares and expresses different proteins like humans, one of which is the ACE-2 protein used by SARS-CoV-2 to infect cells, this protein is not expressed in mice.

Myllymäki et al. [51] evaluated the immunization of adult zebrafish for the preclinical screening of DNA-based vaccines. The authors injected the antigens with the fusion of a GFP protein that allowed the confirmation of the expression of the antigens under UV light, this model allows to evaluate and quantify the systemic levels in vivo. Compared to preclinical mammalian screening models, this method is relatively more economical for preliminary screening of new vaccine candidates. Figure 7 presents a representative scheme of these screenings for the selection of possible vaccine antigens.
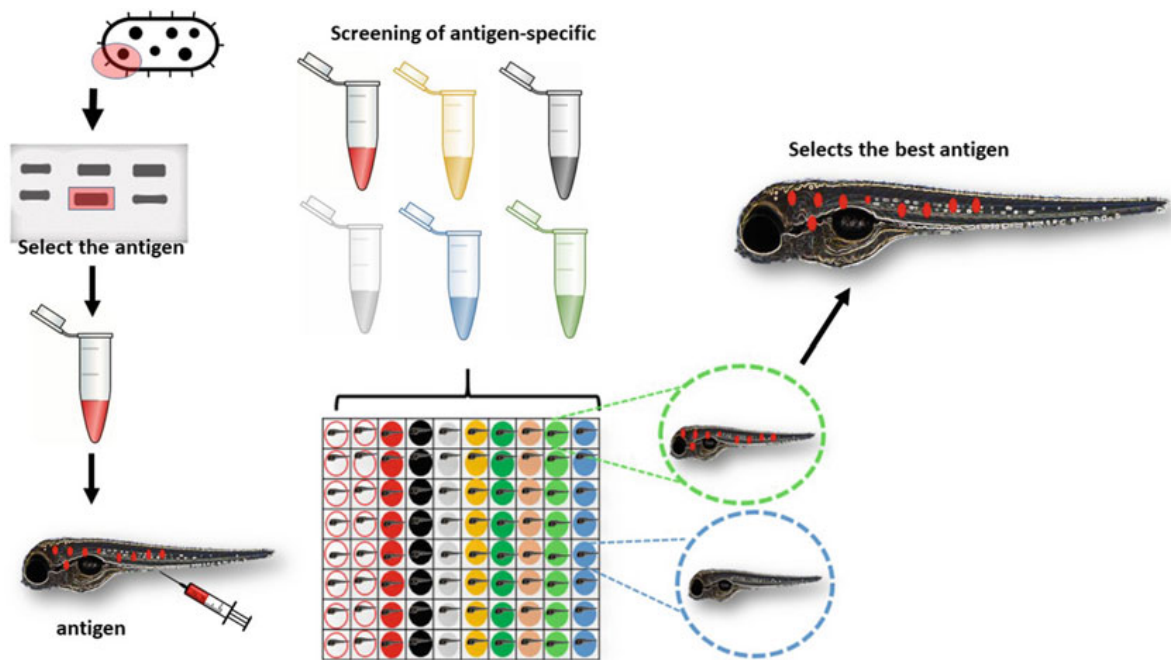
Fig. 7 Representative scheme of these screenings for the selection of possible vaccine antigens
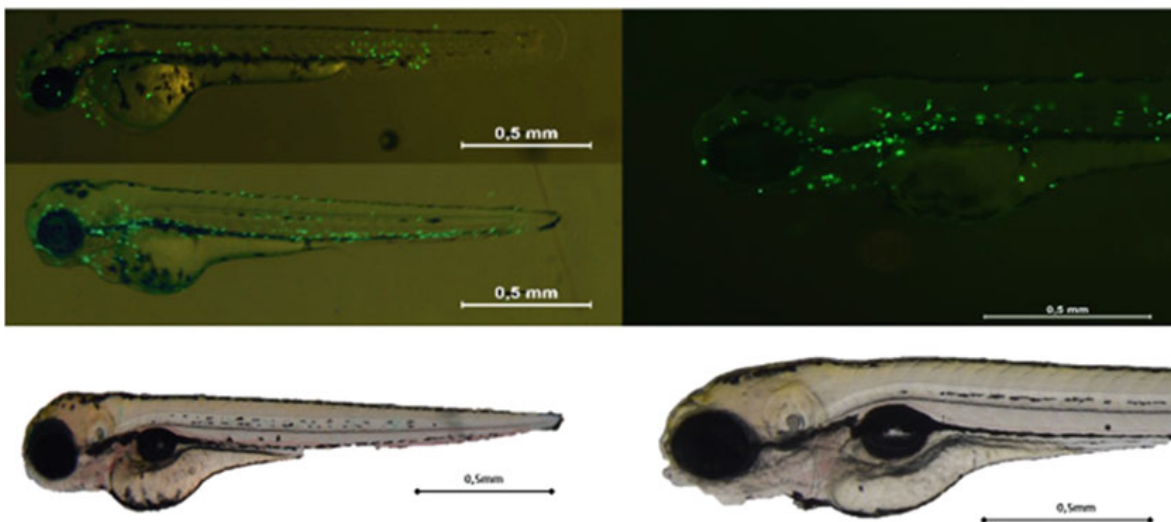
Fig. 8 The MPO: GFP construct drives fluorescent protein expression in neutrophils. (These images of MPO animals were donated by Prof. Dra, Natália Martins Feitosa Integrated Laboratory of Translational Bioscience (LIBT), Institute of Biodiversity and Sustainability (NUPEM), Federal University of Rio de Janeiro (UFRJ)—Macaé, RJ, Brazil)

The optical transparency of zebrafish and the availability of strains with cells marked by MPO-fluorescence provide attractive opportunities for understanding immunological mechanisms and identifying new therapies (Fig. 8), which cannot be obtained with experimental models using rodents.

Another scientific improvement using the zebrafish model was in the development of a vaccine against bovine tuberculosis. The results showed that *M. bovis* protected zebrafish against mycobacteriosis caused by low and high doses of *M. marinum* infection and provided evidence suggesting that the protective mechanism triggered by *M. bovis*, as in other species, is based on the activation of the complement system C3 [52]. After an immunoproteomic analysis of *Streptococcus agalactiae*, a study with the use of chimeras was performed to identify antigenic proteins and build a chimeric multiepitopic vaccine to improve the immune response in another teleost fish, tilapia [53]. Vaccine strategies for several other pathogens have been studied in zebrafish, such as *Salmonella*, which can enhance the dynamics of new vaccine development, and thereby reduce the indiscriminate use of antibiotics in animal production for human consumption [54].

Outer membrane vesicles (OMVs) of Gram-negative bacteria have been used successfully to vaccinate against intracellular and extracellular pathogens, due to the ability to stimulate innate immune responses, the vesicles, when used as a vaccine, reduced the proliferation of the bacterium and protected zebrafish when subsequently challenged with a high dose of *Francisella noatunensis* without causing adverse effects to the host [55].

Another important point to be discussed in fish models to study the development of vaccines is the need for studies relating to the effect of immunizers in different sexes [56]. In general, immunization could be affected by sex, and is more potentiated in females when compared to males in mammals. There is a gap that needs to be clarified in fish, humans, and mice in relation to sex influence. Another insight into human immunization that can use fish as an experimental model is to try to identify the correlation between the early expression of TLR5 and the magnitude of the antibody response in relation to the intestinal microbiota. These innovations are already being reported in other models, in which vaccination of TLR5 mice $(-/-)$ resulted in reduced antibody titers demonstrating an important role of TLR5 in immunity. This was due to a failure to detect the host's microbiota. Thus, antibody responses in mice without germs or treated with antibiotics were impaired. Future studies of gene activation and the role of the microbiota in fish models should also be conducted to assess the influence of gene activation and the response on antibody production [57].

3.1 Vaccine Delivery System

Currently, several studies have investigated the effect of vaccination using various types of antigens. Despite their proven efficacy, their uses are still limited due to the low rate of antibody production. In order to improve vaccine efficacy, nanotechnology has been studied for "deliver system" that involves the study of natural or synthetic particulate material with numerical distribution and dimensions
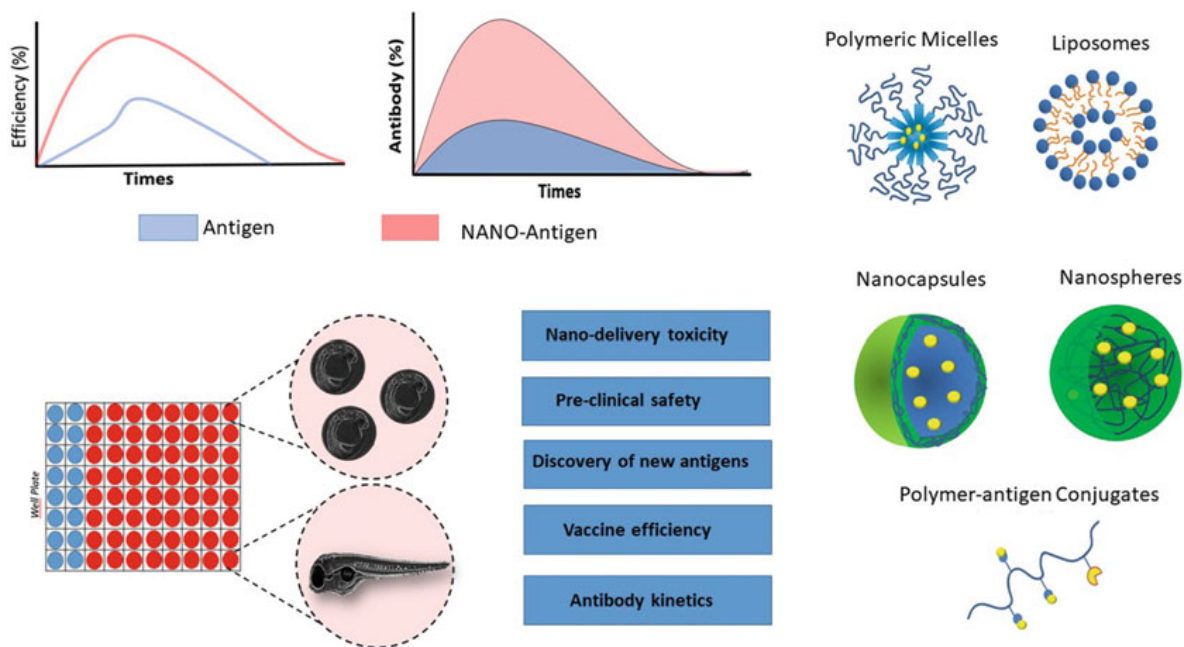
Fig. 9 Schematic representation of studies with different nanocarrier strategies used to improve the delivery of vaccine antigens

between 1 and 100 nm, constituting nanostructured systems [58]. The key points are to improve the solubility of substances and their bioavailability, as well as to protect against degradation, increasing the half-life and its therapeutic efficacy [59]. Here, we highlight only the insights in the development of nanocarriers containing antigens and strategies to improve effectiveness in vivo using zebrafish as an experimental model. In addition, we provide a prospect of the future use of nanocarriers containing antigens, improving the immunomodulatory effects of these compounds. Different types of "nano-delivery systems" have been used to encapsulate antigens such as magnetic nanoparticles; fullerenes); nanostructured lipid carriers; solid lipid nanoparticles; carbon nanotubes; polymeric micelles; nanocapsules; nanospheres; and liposomes (Fig. 9).

Lipid nanoparticles are currently the newest strategies for delivering bioactive molecules from the encapsulation of gene therapies and vaccine development [60]. Weng et al. [61] used lipid-based nano-systems to deliver mRNA molecules in vivo as a new vaccine candidate. The use of fish to study the development of vaccines for aquaculture has been a very promising strategy with the potential to substantially improve the development of effective vaccines for farmed fish and translate these findings. Research on the delivery of viral vaccines using nanoparticles is being a very important milestone in fish vaccinology. In this context, more traditional biomaterials such as alginate have shown good results, however new materials such as solid lipids can improve the delivery of DNA vaccines [62].

There is a need to increase the effectiveness of vaccines in the industry, although several nanoparticle-based vaccine formulations have been reported, as far as we know, only one of them has been implemented in the industry. Kavaliauskis et al. [63] developed and tested zebrafish recombinant gpG poly (I: C) or chitosan-poly (I: C) NPs that could be used as a nonspecific adjuvant in antiviral vaccines. These authors suggest that chitosan-poly (I: C) NPs are a promising adjuvant candidate for future vaccine formulations. Mucoadhesive polymeric nanocapsules have aroused the interest of researchers from different areas of the natural sciences due to their ability to interact with the mucosa and increase the permeation of bioactive substances [64]. In a recent study by our research group, we described a method developed to prepare and characterize mucoadhesive PLGA nanoparticles coated with chitosan and we investigated the interaction of the mucoadhesive system with the fish mucosa, we measured the possible toxic effects on embryos and adults exposed to nanoformulations.

Nanoparticles for the release of immunomodulatory substances in fish have been widely studied by several research groups. We report for the first time a nanostructured system composed of proteins obtained from the mucus of the pacamã fish (*Lophiosilurus alexandri*). We have identified and characterized the formation of nanoparticles [65]. These characteristics suggest a possible application of this material as a biocompatible coating. These nanoparticles are biodegradable, metabolizable and can be easily manipulated; as well as they can easily undergo surface modification to transport biomolecules, thus exhibiting greater versatility. Our study suggests that these fish mucus nanoparticles can be used to release antigens and modulate the immune system, since they have been shown to be a biocompatible coating.

This study opens perspectives for the use of nanoformulations for future studies on mucosal immunity. Our research group has advanced to identify this interaction of nano particles and mucosa, to investigate a new tool for the modulation of fish immune system. We produced a nanoparticle with a fluorescent target and observed an increase in fluorescence over time and mapped where the delivery site would be and the main organs were the spleen, followed by the liver and gills. Taking into account that the spleen participates in adaptive immunity of zebrafish, we believe that our nanoparticles reached target organs. In addition, the use of mucoadhesive nanocarriers becomes an alternative for administration of immunomodulators in immersion systems, since the nanosystem can adhere to the mucosal surface of fish with little residual effect on water [66]. This mucoadhesive nanostructure after immersion in Nile tilapia proved to be very safe without any changes in hemogasometric variables, and its use did not result in mortality [67]. Our research group has studied the toxicity of various types of nanocarriers and microcarriers in fish such as Doxorubicin-loaded

pH-sensitive micelles [68], Mucoadhesive nanocapsules [64]; microplastics [69]; polylactic acid biomicroplastic [70]; Chitosan-coated zein nanoparticles [67]; nanocapsules of poly–ɛcaprolactone containing artemisinin [71].

Another interesting approach in a delivery system is to try to understand the organism's response to nanotechnologies. Crecente-Campo et al. [72] has developed a nanoparticle designed intentionally to target zebrafish macrophages and modulate its response in vaccine development. The results showed that the nanoparticles interacted more efficiently with macrophages in transgenic zebrafish. These authors emphasize that small changes in the nanometric range can lead to a remarkably different interaction with the immune system cells and their biodistribution.

In zebrafish, little is known about the mechanisms of absorption, transepithelial transport and immune response to nanoparticles. Løvmo et al. [73] demonstrated for the first time the absorption of different PLGA nano and microparticles in the intestine and their interactions with epithelial cells and the mucosal immune system. These authors used fluorescent particles or bacteria that were delivered directly to the adult zebrafish's intestine by oral intubation, and their location was photographed in the intestine, liver, and spleen. It was demonstrated that the nanoparticles were quickly captured in the intestine and transported to the liver and spleen. In each tissue, both bacteria and nanoparticles were widely located in leukocytes (presumably macrophages), demonstrating a possible use for oral vaccine delivery studies and showing the nanoparticle's ability to deliver antigens orally.

4   Zebrafish (*Danio rerio*)

Danio rerio (zebrafish) is a freshwater teleost fish in the family Cyprinidae and order Cypriniformes. There are 44 species of the genus *Danio*, all native to southeastern and southern Asia, distributed mainly in northeastern India, Bangladesh and Myanmar. The name *Danio* comes from the Bengali word dhani, which means rice field, considered a gregarious species, normally found in schools of 5–20 individuals of both sexes. Although they are social fish, they can have agonistic behavior, especially during spawning and the establishment of the dominance hierarchy, which occurs between the sexes. However, they are easily kept in captivity with high number of animals reared in small areas, and at much lower operating costs when compared to rodents [74].

Females spawn every 5–7 days, laying 200–300 eggs per week, and reproduce throughout the year [75]. The eggs have an average diameter of 0.7 mm at the time of fertilization, the embryos are transparent, and the fertilization is external, which facilitates the study and manipulation of embryos in the stages of larval

development [75]. The high reproductive capacity containing a significant number of offspring by spawning, minimize the genetic variability within the studies, allowing the development of designs that minimize the experimental statistical errors, helping in the comparison of different treatments effects, increasing the reliability of the studies [76].

There are many questions about human immunization that are difficult to investigate in rodents, but they can be examined using zebrafish as a larval host. The eggs are fertilized and develop into transparent embryos, allowing the observation of organogenesis and phenotypes. Embryogenesis occurs *ex vivo* and is complete 3 days after fertilization, with most organs (e.g., blood vessels, brain, heart, liver, intestine and eyes) being developed within 24 h and becoming fully functional in 1 week [77]. This fact represents a great advantage, since it streamlines actions and speeds up the realization of screenings for the development of vaccines and studies with immunomodulatory substances, increasing the replicability of the models.

The *Danio rerio* genome has been fully sequenced, containing 25 pairs of chromosomes with 26,000 protein coding genes. According to Howe et al. [78], the nucleotide sequence of zebrafish genes has approximately 70% homology with that of human genes, and 84% of the genes known to be associated with human diseases have counterparts in zebrafish, a fact that has been strengthening the use of this model in immunology and applied pharmacology [79]. Therefore, associated with advances in high resolution quantitative imaging and the development of molecular biology, the high prolificity of zebrafish, embryo transparency, lower cost of breeding and the possibility of genetic manipulation of these animals have strengthened its use as an experimental model to investigate mechanisms of immune responses, studies of new drugs and vaccine strategies.

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Chapter 11

Development of Nano-Conjugated DNA Vaccine Against Edwardsiellosis Disease in Fish

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Abstract

Biotechnological advancements have paved newer avenues for developing and designing novel and effective vaccines for rendering protection from various types of infectious diseases. Use of immunogenic genes via plasmid DNA constitutes an important next-generation biotechnological approach to fish immunization. In addition, the use of nanotechnology has significantly addressed the issue of mucosal mode of DNA vaccine delivery in aquaculture. Taking together both these advance technologies, this chapter entails a detailed protocol for the development of a nano-conjugated bicistronic DNA vaccine using chitosan nanoparticles as delivery vehicle, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of *Edwardsiella tarda* as antigenic gene and interferon gamma (IFN-γ) gene of *Labeo rohita* as molecular adjuvant.

Key words Chitosan, Nanoparticles, DNA vaccine, *Edwardsiella tarda*

1 Introduction

Nano-conjugation of DNA vaccine is considered to be an important strategy for development of fish mucosal vaccines. This type of vaccination strategy cumulates of 2 components of advanced vaccine technology—DNA vaccine and nano-conjugation. DNA-based immunization involves the delivery of plasmid DNA (raised in microorganisms such as bacteria) encoding a vaccine antigen to the host. Under the control of eukaryotic promoters, the plasmid DNA (pDNA) expresses inside the recipient, first by transcription into mRNA and then by translation into the protein encoded by the gene. The expressed antigenic proteins are recognized by the host immune system as "foreign," inducing strong and long-lasting humoral and cell-mediated immune responses without the risk of inadvertent infection. Whereas, nano-conjugation involves covalent cross-linking of the pDNA to the surface of nanoparticles which helps in providing stability to the pDNA as well as facilitates

sustained release of the antigen that helps to induce the immunos-timulatory properties of the vaccine.

Edwardsiella tarda (*E. tarda*) is a Gram negative, facultative anaerobic bacterium belonging to the family Enterobacteriaceae, causes Edwardsiellosis/putrefactive systemic infection in both marine and freshwater fishes. In an attempt to control the spread of this disease in Indian major carp, *Labeo rohita*, we constructed a bicistronic DNA vaccine having an antigenic gene (GAPDH of *E. tarda*) and an immune adjuvant gene (IFN-γ of host fish, *L. rohita*) [1] and subsequently conjugated with chitosan nanoparticles (CNPs) for mucosal (oral and immersion) immunization [2]. The present chapter describes a detailed protocol for the same.

2   Construction of Bicistronic DNA Vaccine (pGPD + IFN)

2.1   Designing of Primers for GAPDH Gene of E. tarda and IFN-γ Gene from L. rohita

1. Sequences for GAPDH gene (996 bp) of *E. tarda* (Gene bank accession no. FJ605131.1) and IFN-γ gene (552 bp) of *L. rohita* (Gene bank accession no.HQ667144.1) were retrieved from NCBI nucleotide database.

2. From the sequences, respective open reading frames (ORFs) were found out using "ORF finder" online platform.

3. Primers for both the genes were designed from the ORF sequence by using "Primer Express" software.

4. Compatible restriction enzymes (RE) were selected for both the genes, from pIRES expression vector map for directional cloning.

5. The compatibility of restriction enzyme (RE) with the gene sequences were checked in "NEB cutter" online platform.

6. *Xho I* and *Mlu I* restriction enzymes were selected for forward and reverse primers respectively, for GAPDH gene, whereas; *Sal I* and *Not I* restriction enzymes were selected for forward and reverse primers respectively, for IFN-γ gene (Table 1).

2.2   Isolation of Genomic DNA from E. tarda

1. *Edwardsiella tarda* ATCC® 15,947™ was revived in brain heart infusion (BHI) broth from cult loop. The broth was incubated for 18–24 h at 37 °C.

2. The genomic DNA from freshly cultured *E. tarda* was isolated by using GenElute Bacterial Genomic DNA kit (Sigma, USA) according to the manufacturer's protocol.

3. Concentration of DNA obtained was measured using Nano-drop (Thermo Scientific, USA) and the resultant DNA was stored at −20 °C.

Table 1
Primers designed for GAPDH gene of *E. tarda* and IFN-γ gene from *L. rohita*

Gene (accession no.)	Oligo name	Sequence (5′–3′)	RE site
GAPDH (FJ605131.1)	GPD/pIRES/F	CCC**CTCGAG**ATGACTATCAAAGTAGGTATCA	*Xho* I
	GPD/pIRES/R	CCC**ACGCGT**TTACTTAGAGATGTGTGCGA	*Mlu* I
IFN-γ (HQ667144.1)	IFN/pIRES/F	CCC**GTCGAC**ATGATTGCGCAACAAACAATG	*Sal* I
	IFN/pIRES/R	CCC**GCGGCCGC**TCAAGACTTCTGATTCTTTTTG	*Not* I

***2.3 Extraction of GAPDH Gene from Genomic DNA of* E. tarda**

1. The genomic DNA prepared from *E. tarda* was amplified with specific primer sets for GAPDH gene (996 bp) (Table 1).

2. Bulk PCR was performed using 4×25 μL reaction volume, each containing 2 μL (100 ng) of template DNA, 2.5 μL of $10\times$ PCR buffer (Thermo fisher Scientific, USA), 2 μL of 25 mM MgCl$_2$ (Thermo fisher Scientific, USA), 0.5 μL of 10 mM dNTP mix (Thermo Fisher Scientific, USA), 0.25 μL of Taq DNA polymerase (5 U) (Thermo Fisher Scientific, USA), 0.5 μL each of forward and reverse primers (25 pmol) and rest nuclease free water (Thermo Fisher Scientific, USA).

3. Amplification of GAPDH gene was carried out with following cyclic condition in a thermal cycler (Applied Biosystems, USA): initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min, then a single step of final extension at 72 °C for 10 min.

4. Amplified PCR product was run in 1.2% agarose gel.

5. After running the PCR product in gel, the fragment was excised out of the gel using a coverslip in UV-transilluminator.

6. The gel slice was taken in a pre-weighed 1.5-mL microfuge tube.

7. The gel slice containing DNA fragment of GAPDH gene was subsequently purified by gel extraction using Fermentas Gene-JET™ Gel Extraction kit (Thermo Fisher Scientific, USA).

8. To the gel fragment, binding buffer at 1:1 (w/v) was added and incubated at 55 °C for 10 min for solubilization.

9. The solubilized gel solution was transferred to the GeneJet purification column supplied with the kit and centrifuge for 1 min at $12,000 \times g$. Flow-through was discarded.

10. Column was washed twice using 700 μL of wash buffer containing ethanol and centrifuged for 1 min.

11. To remove residual wash buffer, one additional centrifugation was carried out.

12. The column was then transferred into a fresh 1.5 mL micro-centrifuge tube and 20 μL of elution buffer was added and after incubation for 2 min at room temperature, the DNA was eluted by centrifugation for 1 min at $12,000 \times g$.

13. Concentration of elute was checked using Nano Drop (Thermo Fisher Scientific, USA).

2.4 Extraction of IFN-γ Gene from L. rohita

1. *L. rohita* fingerling was intraperitoneally injected with 100 μL of Poly I:C (100 mg/mL in HBSS solution) for IFN-γ mRNA induction.

2. The kidney tissue was collected at 48 h post-injection in 2 mL homogenization tubes containing glass beads and 1 mL Trizol™ reagent (Invitrogen, USA) for RNA isolation.

3. The samples were homogenized using Micro Smash MS-200 (Tomy, Japan) for 20–30 s at 4000 rpm.

4. The homogenate was incubated for 10 min at room temperature to allow lysis followed by addition of 200 μL of chloroform with vigorous mixing resulting in no separate layers and incubated for 2 min at room temperature.

5. The sample was then centrifuged at $12,000 \times g$ for 10 min at 4 °C.

6. The upper aqueous layer containing RNA was transferred carefully into a fresh 1.5-mL microcentrifuge tube containing 500 μL of isopropanol and incubated for 10 min at room temperature for precipitation of RNA followed by centrifugation at $12,000 \times g$ for 10 min at 4 °C to get the RNA pellet.

7. The RNA pellet was washed twice with 75% ethanol (chilled) by centrifugation at $12,000 \times g$ for 2 min at 4 °C.

8. The pellet was air dried to remove residual ethanol and subsequently dissolved in 30 μL of DEPC water and stored at -80 °C until used for cDNA synthesis.

9. The concentration of the isolated RNA was measured using Nanodrop (Thermo Fisher Scientific, USA).

10. Total RNA isolated using Trizol™ reagent was treated with RNase free DNase l (Fermentas, USA), before cDNA synthesis to remove DNA contamination.

11. The reaction mixture was prepared by adding 2500 ng/μL of total RNA, 2 μL of 10× reaction buffer, 2.5 μL DNase I enzyme in a PCR tube. The final volume was made to 18 μL with NFW.

12. The reaction mixture was incubated for 30 min at 37 °C in a thermal cycler (Applied Biosystems, USA) and subsequently terminated by incubating with 2 μL of 50 mM EDTA at 65 °C for 10 min.

13. The DNase-treated RNA (1 µg) was reverse-transcribed into first-strand cDNA using First-strand cDNA synthesis kit (Thermo Fisher Scientific, USA).

14. The DNase-treated RNA was diluted up to 10 µL with nuclease free water and mixed with 1 µL of oligo-dT primer in a PCR tube.

15. This mixture was incubated at 65 °C for 5 min in a thermal cycler and immediately chilled on ice for 5 min.

16. The reaction volume was made up to 20 µL by adding 4 µL of 5× reaction buffer, 2 µL of dNTP mix (10 mM each dNTP), 1 µL of RiboLock RNase inhibitor (20 units/µL) and 2 µL of Revert Aid M-MuLV Reverse Transcriptase enzyme (20 units/µL), mixed gently by tapping, spun and incubated at 37 °C for 60 min.

17. The reaction was terminated by deactivating the enzyme at 70 °C for 5 min.

18. The resultant cDNA was used as template for extraction of IFN-γ gene.

19. The IFN-γ gene (552 bp) was amplified with designed primer sets (Table 1) in a 25 µL reaction with Taq DNA polymerase (5 U) (Thermo Fisher Scientific, USA) similar to GAPDH gene.

20. The cyclic condition for amplification of IFN-γ was 30 cycles of denaturation (94 °C, 30 s), annealing (65 °C, 40 s) and extension (72 °C, 1 min) with a further final extension (72 °C, 10 min).

21. Amplified PCR product was run in 1.2% agarose gel and subsequently purified by gel extraction using Fermentas GeneJET™ Gel Extraction kit (Thermo Fisher Scientific, USA) as described above for GAPDH gene.

2.5 Cloning of GAPDH Gene and IFN-γ Gene in pTZ57R/T Cloning Vector

1. The required volume of gel extracted GAPDH and IFN-γ genes product were calculated and subsequently cloned into pTZ57R/T vector, using TransformAid Bacterial Transformation Kit (Thermo Fisher Scientific, USA).

2. 3 µL of the pTZ57R/T vector, 6 µL of 5× ligation buffer and 1 µL of T4 DNA ligase enzyme and nuclease free water (upto 30 µL) were added with the eluted product in a 0.2 mL PCR tube.

3. This mixture was incubated at 4 °C for overnight.

4. On same day, freshly streaked *Escherichia coli* DH5-α strain was inoculated in 5 mL of LB broth and incubated at 37 °C in a shaking incubator for 16 h.

5. From the broth culture, 200 µL was inoculated in 1 mL of pre-warmed C-medium, supplied with TransformAid Bacterial

Transformation Kit (Thermo Fisher Scientific, USA) and incubated at 37 °C in a shaker for 4 h.

6. For transformation, 200 μL of the above bacterial culture was again incubated in 1 mL of pre-warmed C-medium for 20 min at 37 °C in a shaker.

7. Cells were harvested by centrifugation at 7500 × g for 2 min and the supernatant was discarded.

8. The bacterial cell pellet was resuspended in 200 μL of T-solution, prepared by mixing 140 μL each of T-solution A and T- solution B (provided with the kit) and incubated for 5 min in ice followed by centrifugation at 7500 × g for 2 min.

9. The supernatant was discarded and pellet was again resuspended in the remaining 80 μL of T-solution and incubated for 5 min in ice thereby, forming competent cells.

10. 5 μL of ligation mixture was added to a fresh microcentrifuge tube and chilled on ice for 2 min; to it 50 μL of the prepared competent cells was added and incubated on ice for 5 min.

11. The transformed cells were immediately spread on LB agar plates containing ampicillin (100 μg/mL) and 40 μL each X-gal (20 mg/mL) and IPTG (100 mM) under sterile conditions and incubated at 37 °C for 12 h.

12. Recombinant clones grown on LB-ampicillin (100 μg/mL) agar were identified by blue-white screening.

13. Single colony of recombinant clone were and inoculated in 5 mL LB broth containing ampicillin (100 μg/mL) at 37 °C for 16 h.

14. The recombinant plasmid DNA was isolated using the GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific, USA).

15. Bacterial cells were harvested by centrifugation at 6000 × g in a microcentrifuge for 2 min.

16. Bacterial pellet was resuspended in 250 μL of Resuspension solution and mixed thoroughly.

17. 250 μL of Lysis solution was added and mixed by inverting the tube five times.

18. To the mixture, 350 μL of neutralization solution was added and mixed properly followed by centrifugation at 11,000 × g for 5 min.

19. The supernatant was transferred to the supplied GeneJET spin column without disturbing white precipitate.

20. After centrifugation at 11,000 × g for 1 min the flow-through was discarded.

21. 500 μL of Wash solution was added to the column and centrifuge for 1 min; washing process was repeated.

22. The column was transferred to a fresh 1.5 mL microcentrifuge tube and 20 μL of NFW was added to elute the plasmid by centrifugation for 2 min.

23. The plasmid was stored in −20 °C after checking the concentration of the recombinant plasmids using Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) in terms of ng/μL.

2.6   Cloning of GAPDH Gene and IFN-γ Gene in pIRES Expression Vector

1. GAPDH gene from pTZ57R/T was released by digestion of recombinant plasmid using restriction enzyme *Xho* I and *Mlu* I. Simultaneously pIRES vector (6.1 kb, Clontech, USA) was digested with same restriction enzyme.

2. The restriction digestion protocol is as follows: 9 μL of plasmid DNA, 6 μL each of *Xho* I and *Mlu* I, 6 μL 10× Fast digest buffer, remaining NFW (upto 60 μL) were added in PCR tube and incubated at 37 °C for 15 min followed by 5 min incubation at 80 °C.

3. The digested products (GAPDH insert and pIRES vector) were run separately in 0.8% agarose gel and purified using Fermentas GeneJET™ Gel Extraction kit (Thermo Fisher Scientific, USA) as described above.

4. Thereafter the GAPDH gene was cloned in pIRES vector in Frame A using TransformAid Bacterial Transformation Kit (Thermo Fisher Scientific, USA) as previously described and designated as pGPD.

5. Similarly, IFN-γ gene from pTZ57R/T was released by digestion of recombinant plasmid using restriction enzyme *Sal* I and *Not* I and subsequently cloned in pGPD in Frame B after digestion with same restriction enzyme.

6. The resultant plasmid vector containing both GAPDH and IFN-γ gene was designated as pGPD+IFN (Fig. 1).

3   Extraction of Plasmid DNA Construct (pGPD + IFN)

1. Plasmid (pGPD/IFN) extraction was done by using QIAGEN Plasmid Maxi Kit (QIAGEN, Germany).

2. Single colony of freshly streaked plate is inoculated in 5 mL LB-ampicillin broth and incubated at 37 °C in shaking incubator overnight.

3. On next day 300 μL of the culture is inoculated in 300 mL of LB-ampicillin broth and incubated at 37 °C in shaking incubator for 12–16 h followed by harvesting of bacterial cells by centrifuging at $6000 \times g$ for 15 min at 4 °C.
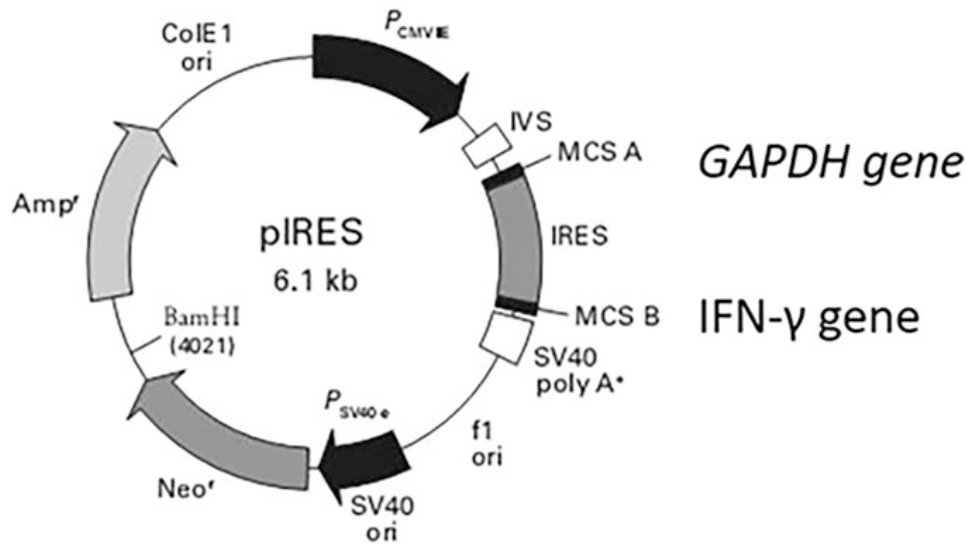
Fig. 1 Schematic diagram of the constructed bicistronic DNA vaccine; GAPDH gene and IFN-γ gene cloned in the eukaryotic expression vector, pIRES vector (6.1 kb having CMV promoter) at Frame A and Frame B, respectively

4. The bacterial pellet was resuspended in 24 mL buffer P1, to it 24 mL of P2 buffer was added and mixed thoroughly by gently inverting the tube four to six times, and then incubated at room temperature (15–25 °C) for 5 min.

5. Subsequently, 24 mL of prechilled P3 buffer was added and mixed thoroughly by inverting the tube four to six times upside down followed by incubation on ice for 20 min.

6. The mixture was then centrifuged at $17,000 \times g$ for 40 min at 4 °C.

7. The supernatant was then filtered and collected in 50 mL centrifuge tube.

8. The QIAGEN-tip500 column was equilibrated with 10 mL of equilibration buffer (QBT).

9. The collected supernatant was poured into column and allowed to empty by gravity flow resulting in entrapment of the plasmid DNA in the resin column.

10. The column was washed twice by 30 mL of wash buffer (QC); the wash buffer was allowed to move through the column by gravity flow.

11. The pDNA was eluted with 15 mL of pre-warmed elution buffer (QF) into a clean 50 mL tube.

12. The pDNA was precipitated by adding 10.5 mL (0.7 volumes) of isopropanol at room temperature and centrifuged at $15,000 \times g$ for 30 min at 4 °C.

13. The supernatant was carefully decanted and the glassy pDNA pellet was washed with 5 mL 70% ethanol at room temperature and centrifuged at 15,000 × *g* for 10 min at 4 °C.

14. The pellet was then air dried for 5–10 min and pGPD/IFN was dissolved in 500 μL NFW. Likewise, plasmid extraction was repeated to get sufficient quantity and concentration of plasmid.

15. The concentration of the recombinant plasmids was measured using Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) and diluted with nuclease free water (NFW) to 100 ng/μL concentration and stored at −20 °C.

4   Conjugation of pGPD + IFN with Chitosan NPs

4.1   Preparation of Chitosan Nanoparticles (NPs)

1. 0.2 g of chitosan (Sigma-Aldrich) was dissolved in 10 mL of glacial acetic acid.

2. 60 mL of distilled water was added to it and kept on magnetic stirrer for 3 h with vigorous stirring.

3. After complete dissolution of chitosan, the pH of the solution was adjusted to 5.5 using NaOH and the final volume was made up to 100 mL by adding distilled water.

4. The solution was kept on magnetic stirrer for another 1 h for complete mixing.

4.2   Determination of Zeta Potential and Size of Nanoparticles

1. The prepared chitosan NPs were characterized in terms of size, size distribution, and zeta potential by dynamic light scattering (DLS) using HORIBA Scientific Nano particle analyzer SZ-100 (HORIBA, Japan).

2. 1.3 mL of sample at a concentration of 0.3 mg/mL was placed in a polystyrene cuvette and measured at 25 °C. The viscosity and refraction index were set equal to those specific to water.

3. Zeta potential was measured with a disposable capillary cell with a volume of 1 mL after purification.

4.3   Conjugation of pGPD + IFN with Chitosan NPs

1. Equal volume of chitosan NPs solution (0.02% in 25 mM sodium acetate buffer, pH 5.5) and pGPD+IFN (100 ng/μL) were taken in 15 mL tubes separately and heated to 55 °C in water-bath for 5 min.

2. The heated chitosan was added to the heated plasmid drop-wise.

3. The final mixture was subjected to vortexing at 1000 × *g* for 30 s and kept at room temperature for 30 min.

4. The size and zeta potential of the chitosan NPs conjugated pGPD + IFN (CNPs-pGPD + IFN) was determined as described previously and was stored at 20 °C as the nano conjugated vaccine.

References

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Chapter 12

Development and Evaluation of DNA Vaccine Against Salmonid Alphavirus

Chia-Jung Chang

Abstract

Despite vaccination, pancreas disease (PD) caused by salmonid alphavirus (SAV) has been the economically most important virus disease in salmon farming in Ireland, Scotland, and Norway. A vaccine based on DNA plasmid has been authorized to be used in Norwegian aquaculture since 2018. DNA vaccination of plasmids expressed subcellular viral proteins have been shown its particular protective effect against SAV3 that surface expression of the E2 protein with the whole viral protein construct, yielding a more effective vaccine. The chapter describes methods to design and test the sublocalization of expressed viral protein and the performance evaluation of vaccines against SAV3 infection in Atlantic salmon.

Key words Salmonid alphavirus, DNA vaccine, Pancreas disease, Subcellular antigen expression, ELISA, Neutralizing activity, Histopathology

1 Introduction

Pancreas disease (PD) is caused by salmonid alphavirus (SAV), leading to severe economic loss in farmed Atlantic salmon in Norway, Ireland, and Scotland [1]. In recent years PD is considered one of the most severe virus diseases in Norwegian salmon farming [2]. The fish disease was first recognized and described in Scotland in 1976 [3, 4] and is typically characterized by histological changes, including severe degeneration, necrosis, and inflammation in the pancreas, heart, and skeletal muscle [5–8]. Based on phylogenetic analysis, six subtypes of SAV (SAV1-SAV6) have been identified [9, 10]. Different geographic locations of the PD epidemics are associated with various strains of SAV. SAV1 is the major SAV strain in Ireland and Scotland [1]. SAV2 is the major strain causing epidemic in central Norway, while SAV3 is the cause of the long-lasting PD epidemic in the western Norway [2]. The third subtype of salmonid alphavirus (SAV3) was first identified in 2005 in Norway, which shares 91.6% and 92.9% similarity of its nucleotide

sequence compared to SAV1 and SAV2 [9]. Alphaviruses are enveloped viruses with a genome consisting of one positive sense RNA strand [10]. The structural ORF is encoded by a 26S sub-genomic mRNA, which is translated as a polyprotein that is cleaved into the five structural proteins—capsid (C), E3, E2, 6K, and E1. Recent studies have shown that the processing/trafficking of SAV glycoproteins E1 and E2 depends on low temperature, i.e., 15–18 °C [13, 14], and the conformation dependence of the viral protein assembly was found similar to human alphavirus in the endoplasmic reticulum membrane during the cleavage procedures (Fig. 1a-2). The two glycoproteins, El and E2, are anchored in the membrane and interact to form spikes at the viral surface [10, 11]. It was found that the surface expressed E2 is dependent on the co-expression of E1 [12]. In the DNA vaccine, the plasmid construct expressed the surface E2 protein could trigger antibody response and neutralization activity against SAV3 compared to its intracellular protein [13]. Therefore, the present chapter presents protocols for designing the DNA vaccine constructs depending on the subcellular expression of viral protein and procedures for performing the trial experiments and assessing vaccine efficacy. In general, a semi-quantitative scoring system based on histopathology that has been developed for identifying the PD caused severe degeneration, necrosis, and inflammation in the pancreas, heart, and skeletal muscle [1], which is a promising tool for evaluating protective effect of vaccines. Additionally, the measurement of virus load in the tissues and sera has been developed using qRT-PCR analysis. The protocols to identify the DNA vaccine-induced serology were recently established for measuring SAV3-specific antibody response and virus-neutralizing activity [13].

2   Materials

2.1 DNA Plasmid Preparation

1. *E. coli* for plasmid production.
2. Vector with CMV promoter and terminator.
3. EndoFree plasmid purification kit: commercially purchased.
4. 1% agarose gel.
5. Endotoxin Assay Kits: commercially purchased.

2.2 Transfection and Immunostaining of Antigen Protein

1. CHSE-214 cells: derived from ATCC growth in MEM + 2 mM Glutamine + 1% Non-Essential Amino Acids + 120mg/l Sodium Pyruvate + 10% Fetal Bovine Serum.
2. Transfection reagent: commercially purchased.
3. 24 wells cell culture plate.
4. Fixation reagent for membrane protein: 4% Paraformaldehyde in PBS.
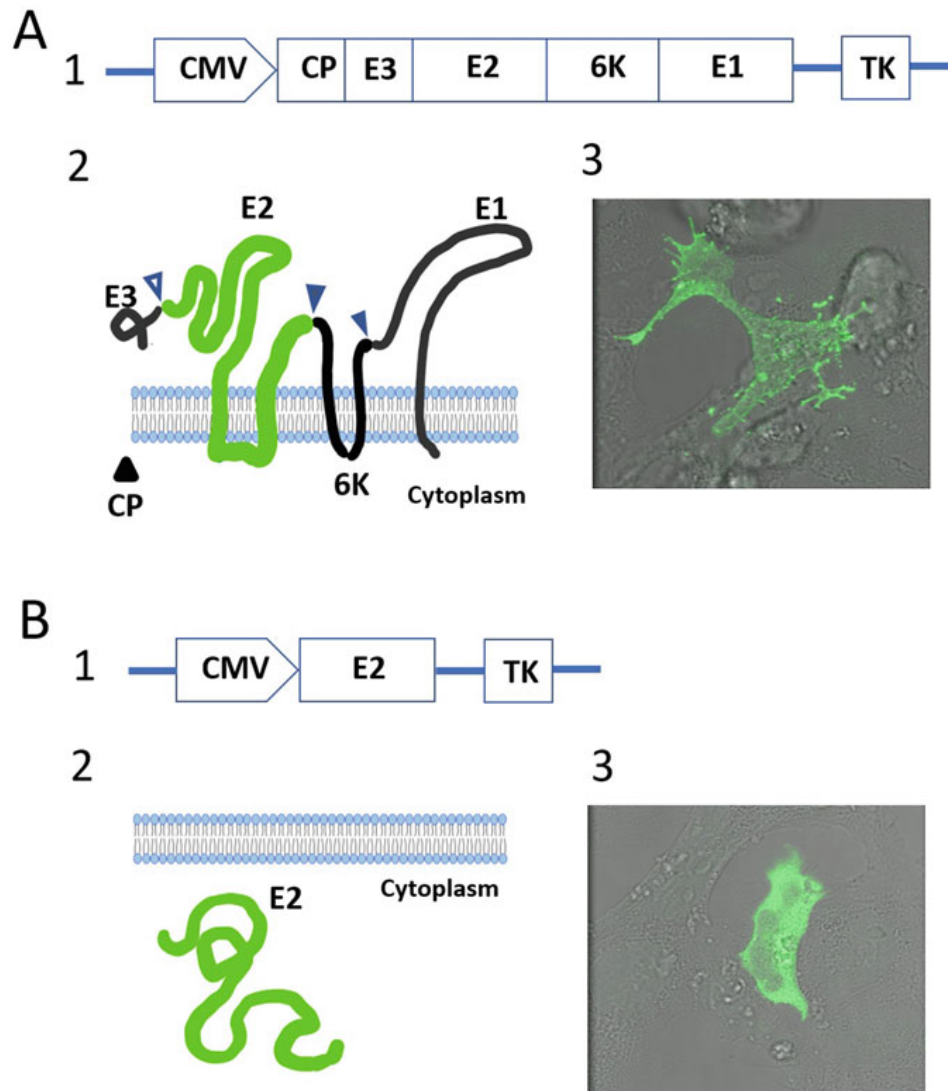
Fig. 1 Example of designing DNA vaccine constructs and the schematic illustration of the topology and staining of virus E2 protein. The DNA vaccine construct with the whole polyprotein that expresses surface E2 protein (**A-1**). Upon translation the capsid (CP) protein is first cleaved and released in the cytoplasm, the N-terminus E3 with signal peptide leads the polyprotein translocation across the membrane. Open and closed arrows indicate the furin and signalase cleavages between each protein, and the remaining proteins are joined through the membrane with the insertion of transmembrane domains (**A-2**) [11]. In contrast, the construct expresses the E2 only (**B-1**) that E2 remained in the cytoplasm post translation (**B-2**). The **B-3** and **B-3** indicated the membrane and intracellular E2 protein staining in plasmid transfected CHSE-214 cells, respectively

5. Fixation reagent for intracellular protein: Acetone:Alcohol, 50%/50% Mixture.

6. Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (D-PBS).

7. Blocking solution: 5% BSA in D-PBS.

8. Primary antibody against salmon alphavirus.

9. Secondary antibody FITC-conjugated: commercially purchased.

2.3 Immunization and Virus Infection

1. Atlantic salmon pre-smolt.
2. 0.5 mL 31-Gauge insulin syringe.
3. D-PBS.
4. Salmon alphavirus.

2.4 Neutralization Activity of Antibody Against Salmonid Alphavirus

1. CHH-1 cells: derived from ECACC growth in L-15 + 2 mM Glutamine + 1% Non-Essential Amino Acids + 10% Fetal Bovine Serum.
2. Maintenance medium for virus culture: L-15 + 2 mM Glutamine + 1% Non-Essential Amino Acids + 2% Fetal Bovine Serum.
3. 96-well plate.
4. Crystal violet solution: 1% Crystal violet in 20% ethanol, pH 7.
5. Solubilizing buffer: 0.05 M sodium citrate + 0.05 M citric acid in 50% ethanol.

2.5 Tissue Sampling for Quantitative Reverse Transcription PCR (qRT-PCR)

1. Sera and heart from virus-infected fish.
2. RNAlater.
3. Homogenizer.
4. Homogenizer beads.
5. RNA isolation kit: commercially purchased.
6. cDNA synthesis kit: commercially purchased.

2.6 ELISA for Antigen-Specific Antibody Response

1. 21-Gauge needle.
2. Nunc MaxiSorp™ flat-bottom 96-well plate.
3. Coating buffer: 0.03 M Na_2CO_3 + 0.07 M $NaHCO_3$ in 1000 mL distilled water, pH 9.6.
4. TBST: 20 mM Tris-base + 150 mM NaCl and 0.1% (w/v) Tween 20, pH 7.4.
5. Antibody against salmonid Ig.
6. HRP-conjugated antibody: commercially purchased.
7. TMB substrate solution (3,3′,5,5′-tetramethylbenzidine): commercially purchased.
8. Stop solution: sulfuric acid (2 M H_2SO_4).

2.7 Histopathology

1. Heart, muscle and pancreas from virus-infected fish.
2. 10% Neutral Buffered Formalin: commercially purchased.
3. Ethanol: different percentage.
4. Tissue embedding medium: mixture of highly purified paraffin containing plastic polymers, commercially purchased.
5. Tissue clearing agent: Histo-clear, commercially purchased.
6. Shandon™ Instant Hematoxylin and Instant Eosin, Alcoholic: commercially purchased.

3   Methods

3.1   Design the Constructs

1. A plasmid construct should include a promoter and a terminator at the N-and C-terminal, respectively, for the gene of interests derived from the virus as Fig. 1a-1 or b-1 (*see* **Note 1**). Besides, a functional Kozak sequence right before the start codon (ATG) and a stop codon at the end should be included in the sequence. It is recommended to test several reporter genes (e.g., GFP) or tags (e.g., Flag-tag) as the fusion protein if the downstream analysis is desired.

3.2   Production and Confirmation of the DNA Vaccines

3.2.1   Plasmid Production and Qualification

1. Plasmid production by *E. coli* was following the manufactory's protocols, and the plasmid quality and quantity was confirmed by nanodrop and agarose gel (*see* **Note 2**).

2. The gDNA and endotoxic contamination were measured by qPCR and commercially available kit (*see* **Note 3**).

3.2.2   Transfection and Surface Protein Staining

1. Upon transfection, the CHSE-214 cells were seeded on the 24-well plate 24 h before transfection. The transfection procedures followed the manufacturer's protocols, and the cells were transferred to 15 °C for incubation. After 72–96 h of transfection, the cells were washed gently twice with medium to remove cell debris. Ice cold 4% paraformaldehyde and acetone/ethanol were added to the cells for fixation at 4 °C for 30 min (*see* **Note 4**). After fixation and washing twice with D-PBS, blocking solution was added to the cells and incubated at 4 °C for 1 h (*see* **Note 5**). After blocking and washing twice with D-PBS, the virus protein was stained with the primary antibody against SAV3 E2 protein at 4 °C overnight (*see* **Note 6**).

2. After overnight incubation of the primary antibody, the cells were washed twice with PBS and stained with fluorescence conjugated secondary antibody at room temperature for 2 h. Cells were acquired on confocal microscopy (Leica TCS SP5) equipped with 488 laser lines.

3. Surface staining of E2 protein was identified as those cells that were fixed by 4% paraformaldehyde in Fig. 1A-3. In contrast, intracellular protein staining were done in cells that were fixed by acetone: alcohol solution, as shown in Fig. 1B-3.

3.3   Salmonid Alphavirus Propagation and Immunofluorescence Staining

1. CHH1 cell was grown at 20 °C, SAV3 virus isolate was propagated and titrated in CHH1 cells with a growth medium in 2% FBS at 15 °C (*see* **Note 7**).

2. After 72 h of virus infection with moi = 0.1, the cells were gently washed twice with PBS and fixed with 4%
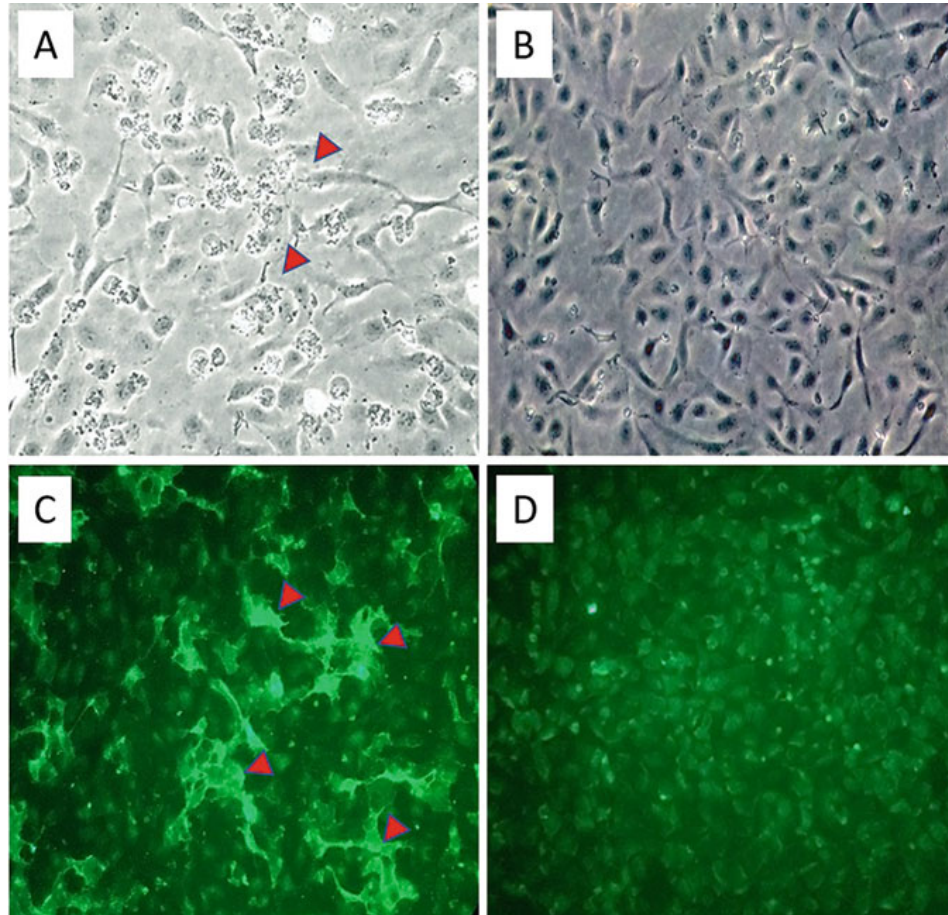
Fig. 2 Salmonid alphavirus infection in CHH-1 cells and immunofluorescent staining of virus E2 protein. The cytopathy effect of virus-infected cells (**A**) and surface virus E2 protein staining (**C**), in contrast to the negative control (**B**, **D**)

paraformaldehyde for surface viral protein staining by following the procedures as described previously (*see* Subheading 3.2.2).

3. Surface staining of alphavirus E2 protein from virus-infected cells is identified and acquired on inverted microscopes (Leica DMi1), as shown in Fig. 2C.

3.4 Schedule for Vaccination, Challenge and Sampling

1. To test the DNA vaccine, the Atlantic salmon pre-smolt around 30 g should be considered, and the fish were kept in tanks supplied with fresh water at 10–12 °C and were fed commercial dry food. The use of smaller size fish was because they require less labor force and easier for the tank arrangements. Besides, the age and water temperature also play a role in the immune response that is normally used for vaccination studies (*see* **Note 8**).

2. Prior to DNA vaccination, the fish were anesthetized with 0.005% benzocaine (ACD Pharmaceuticals, Norway). Fish

Fig. 3 Example of immunization schedule for evaluating a potential DNA vaccine construct of salmonid alphavirus

groups were labeled by tattooing (2% alcian blue, Panjet inoculator) (*see* **Note 9**).

3. The salmon pre-smolts were injected intramuscularly (i.m.) approximately 1 cm below the dorsal fin with one 15 μg plasmids in 50 μL sterile PBS pH 7.4. Sera were collected from 15 fish post ten weeks (Fig. 3) vaccination for measuring antigen-specific antibody titers by ELISA and neutralization assay.

4. As shown in the Fig. 3, after ten weeks of vaccination, the fish were challenged using either virus injection or cohabitant infection by adding 20% shedder fish. The injection method could be done intraperitoneally (i.p.) or intramuscular (i.m.) delivered with 0.1 mL 5000 SAV3 virus particles in each fish. After virus infection, fish were sampled at 7- and 21-days post virus challenge for analysis of virus load in serum (W11, 7 dpi) and histopathology of the pancreas, heart, red and white skeletal muscle (W13, 21 dpi). For the cohabitant infection method, the fish received virus by i.p. are therefore used as the shedders.

5. For the sera collection method, the sera were collected from the caudal vein using disposable syringes with 21-gauge needles and immediately transferred to Eppendorf tubes. The sera were kept at 4 °C and allow to clot for 4 h then clot from serum was separated by centrifugation at $2000 \times g$ for 20 min at 4 °C (*see* **Note 10**). The sera were carefully transferred and aliquoted into small PCR tubes and kept at −80 °C before further analysis.

6. For tissue collection methods, the tissues for histopathology were cut into 5 mm (*see* **Note 11**), transferred into 40 mL formalin (*see* **Note 12**), and incubated on a shaker at low speed at 4 °C overnight. The tissues were then transferred into 40 mL 70% EtOH before embedding (*see* **Note 13**). The tissues for RNA isolation were cut into 300 mg and submerged into five volumes of RNAlater solution (1.5 mL). The tissues in RNAlater were first incubated overnight at 4 °C, then transferred to −20 °C for long-term storage (*see* **Note 14**).

3.5 Evaluation of the Vaccine Efficiency

1. Measurement of antigen-specific antibody response by ELISA assay.

   (a) To measure antibody titers, either purified recombinant SAV3 E2 protein or purified virus can be used as a coating antigen (*see* **Note 15**).

   (b) E2 protein corresponding to 500 ng/ well protein or 200 ng/well purified virus in carbonate coating buffer is coated in 96 well and incubated overnight at 4 °C. After washing three times with TBST, the plate was blocked with 5% skim milk for 2 h at room temperature, then washed three times with TBST before adding fish sera.

   (c) The fish sera were either 50 times diluted or serially diluted with duplicates in TBST and added into the antigen-coated 96 well plates and incubated overnight at 4 °C (*see* **Note 16**).

   (d) After incubation, the plate was washed three times with TBST before applying the secondary antibody against salmonid Ig and incubated at room temperature for 2 h (*see* **Note 17**). The plate was then washed three times with TBST, followed by the addition of the horseradish peroxidase-conjugated third antibody and set at room temperature for 2 h and washed six times with TBST before adding substrates. The reaction is visualized by adding 100 μL/well TMB substrate and incubated in the dark for 10 min. Upon adding 100 μL/well a sulfuric acid stop solution was added before detection at a wavelength of 450 nm.

2. Virus-neutralizing activity.

   (a) To measure the neutralizing activity, 20,000 CHH1 cells/ well were seeded on to 96 well culture plates 1 day before adding the virus.

   (b) The sera with 1:10 to 1:5120 dilution were mixed with 200 SAV3 virus particles in 2% L-15 maintenance media and incubated with CHH1 cells immediately for 2 h at 15 °C. The antibody-virus mixture was replaced with a maintenance medium and incubate for 14 days at 15 °C.

   (c) After 14 days, the supernatant was removed, and the survival cells were determined and stained by adding 100 μL of crystal violet solution and incubated at room temperature for 10 min.

   (d) After staining, the crystal violet was removed, and the plate was washed three times with distilled water and dried at room temperature (*see* **Note 18**).

   (e) The value from stained survival cells is visualized by dissolving the crystal violet by adding 100 μL/well

solubilizing buffer and incubated for 5 min at room temperature on the shaker, prior to detection at a wavelength of 550 nm.

(f) The titers of neutralizing antibody were shown as protection of the highest reciprocal above 50% optical density from non-infected cells using the formula: 50% protection OD = (OD non-infected cells + OD virus-infected cell)/ 2.

3. Virus load in the serum and heart by quantitative RT-PCR analysis.

(a) To measure the virus load from SAV3 infected fish, either sera or heart can be used as the target organs to evaluate vaccine efficacy.

(b) The RNA isolation from the sera sample using the Viral RNA purification kit (Qiagen) was done by following the manufacturer's protocols (*see* **Note 19**). A standard virus curve of ten fold dilutions of known SAV3 virus (10^5 to 10^1 TCID$_{50}$/mL) in 100 μL negative salmon serum was made to establish the correlation between infected serum sample and virus titer (TCID$_{50}$/mL). Virus RNA was then isolated by QIAmp Viral RNA Mini Kit (cat.52906, Qiagen) from 100 μL serum and cDNA were synthesis with cDNA reverse transcription kit according to its manufacturers respectively. Virus load was analyzed using SAV3 specific primers via qPCR analysis. Each individual's CT value was then calculated and transferred to its corresponding virus titer (TCID$_{50}$/mL).

(c) The RNA isolation from the tissues using the RNeasy Mini Kit (Qiagen) was done following the manufacturer's protocols. The tissues were homogenized in the lysis buffer with 1% 2-mercaptoethanol with either ceramic spheres or steel balls. The tissue lysate was mixed with two times volume RNase-free water and proteinase K (*see* **Note 20**) and incubated at 55 °C for 10 min before further purifications.

(d) RNA concentration and quality were determined by Nanodrop and agarose gel electrophoresis, then stored at −80 °C for further RT-qPCR analysis.

4. Histopathology

(a) Histological examinations were carried out by embedding the tissues in paraffin wax before sectioning. The tissues were first dehydrated and infiltrated with molten paraffin wax by standard procedures using a tissue processor (Shandon citadel 1000).

(b) The paraffin infiltrated tissues were embedded and molded into tissue embedding medium and stored in the refrigerator before sectioning.

(c) Prior to sectioning, the paraffin tissue blocks were cooled on ice and cut to produce 3–5 μm thick tissue sections using rotary microtome (Leica RM2235). The sections were transferred into 40 °C water baths and further adhering to the slides (*see* **Note 21**).

(d) All sections were stained with hematoxylin and eosin (H&E) following standard procedures, and coverslip slides using xylene based mounting medium. The histologic examination was done as a blind study as previously described using a semi-quantitative scoring system for evaluating the severity of lesions in the heart, exocrine pancreas, and red and white skeletal muscle was classified as "Normal, Mild, Moderate, and Severe" [14] (*see* **Note 22**).

4   Notes

1. Although the immediate early gene of the human cytomegalovirus was designed for mammalian expression vector, it works perfectly well in many types of fish cells that have been commonly used for fish DNA vaccine studies. Besides, the vector (e.g., pcDNA3.3) containing herpes simplex virus thymidine kinase (TK) polyadenylation signal at the C-terminal has shown that it increases higher expression levels of target protein in fish cell lines compared to the vector (e.g., pcDNA3.1) containing the bovine growth hormone (BGH) polyadenylation signal.

2. Several *E. coli* strains, such as DH5α and TOP10 that have been genetically modified, are commonly used and commercially available for large-scale plasmid production. One thing to be aware that these types of *E. coli* with recA1 genotype may lead the plasmid to form various supercoiled multimeric forms, usually show different patterns in the gel during electrophoresis analysis. The multimeric form of supercoiled plasmid will not influence the transfection efficiency or the biological function in vivo.

3. To test the residues of unwanted gDNA and endotoxin is the standard procedure prior to use the plasmid for in vivo study, which can be measured by qPCR for gDNA analysis [13] and commercial kit such as the gel clot LAL assay for endotoxin measurement.

4. For 1 L of 4% formaldehyde, add 100 mL of 10× PBS in 700 mL distilled water and 40 g of paraformaldehyde powder

and heated on a 60 °C hot plate and stirred in the hood by adding 1 N NaOH dropwise, then cooled and filtered in a 0.45 μm filter. Adjust the volume to 1 L with distilled water and pH to 6.9, then used immediately or aliquoted and frozen at −20 °C for long-term storage.

5. It is recommended to use high purity BSA without IgG contamination as the blocking agent for immunofluorescent staining to reduce the background.

6. Primary antibody staining can be done at 37 °C for 1 h, but a higher background is expected.

7. The propagation of SAV sometimes is tricky due to the low virus titer and without a suitable cell line. Several cell lines, such as CHSE-214, CHH-1, and RTG-2 cells, should be tested for virus isolation, growth, and titration. In particular, SAV3 can be grown in both CHSE-214 and CHH-1 cells, but a visible cytopathic effect can only be observed in CHH-1 cells.

8. Although the "degree days" have been used to calculate the duration needed for the fish experiments, the temperature at 10–12 °C is considered the critical factor for the antibody response. The immune response may absent or occur slowly when the water temperature is below 10 °C.

9. Many methods can be used for fish labeling, including tattooing, fin cutting, and PIT-tag implantation. To be aware that tattoo may disappear, the cut fin will be recovered within a few months. Besides, PIT-tag is more suitable for a fish size bigger than 15 g.

10. Approximately 30–40% sera are expected to be obtained from the blood.

11. When cutting tissues, the whole heart should be harvested or cut into half for improving the infiltration. The muscle should be cut carefully to include both white and red muscle, and the tissue of the pancreas should consist of fat tissue and pancreatic duct.

12. It is essential to use adequate volume of formalin (1:40) for tissue infiltration. Besides, 4% paraformaldehyde in PBS can also be used for tissue fixation.

13. It is essential to have a sufficient amount of formalin and ethanol for tissue infiltration. If the tissues are intended to be used for immunohistochemistry, then the formalin should be replaced by ethanol after 24 h incubation to prevent over fixation.

14. A sufficient amount with at least five times the volume of RNAlater is critical for preserving RNA degradation, and the tissue in RNAlater should not be stored in the freezer immediately; the RNAlater should allow being infiltrated into the

tissue overnight at 4 °C. Besides, if handmade RNAlater is desired, then DEPC-treated water and high-quality chemicals without RNase contamination should be used.

15. The signal peptide was removed for E2 protein production, and the codon usage was optimized and synthesized by the company for *E. coli* expression. The E2 protein was expressed as an inclusion body and purified with 6 M urea and his-tag column and refolded by dialysis in phosphate buffer saline (D-PBS) and store at −80 freezer. Purified E2 protein was further used for antibody production in rabbit and ELISA assay. Except for using recombinant E2 protein, purified SAV3 can be used as the coating antigen for ELISA. SAV3 produced by CHH-1 cells can be purified by polyethylene glycol purification combined with sucrose gradient ultracentrifugation, approximately 1 mg virus protein can be obtained from 1 L high titer virus supernatant.

16. It is preferable to incubate the fish sera with coated antigen at 4 °C overnight to obtain a stronger signal and lower background.

17. Many anti-salmonid Ig antibodies are commercially available, whereas these antibodies need to be tested in advance (i.e., dilution fold) according to various purposes (i.e., ELISA, western blot).

18. Cotton swab that was infiltrated with solubilizing buffer was used to remove the excess crystal violet on the wall of the wells.

19. Although RNA isolation can be done using the traditional Trizol method, the most effective method is to use commercially available kits for large samples that produce less biohazards. The purpose of establishing the correlation of SAV3 virus titer with infected fish in the sera is also to confirm the purification procedures and quantification of virus load for calculation.

20. The viral RNA extracted from tissues does not require carrier RNA compared to the sera. Proteinase K is useful for digesting fibrous tissues such as muscle and heart. Besides, the virus genes replicated differently in the tissues (e.g., non-structure protein 1 has lower replication numbers in the cells than the E2 gene), but not for the virus particles in the sera. Therefore, it is critical to choose correct virus-specific primers for quantitative analysis.

21. The tissue sections may be stored on a dry paper at room temperature for long-term storage before adhering to the glass at 60 °C for 30 min.

22. The H&E staining of the pancreas, heart, and red muscle in Fig. 4 demonstrates the healthy tissues and the tissues that have
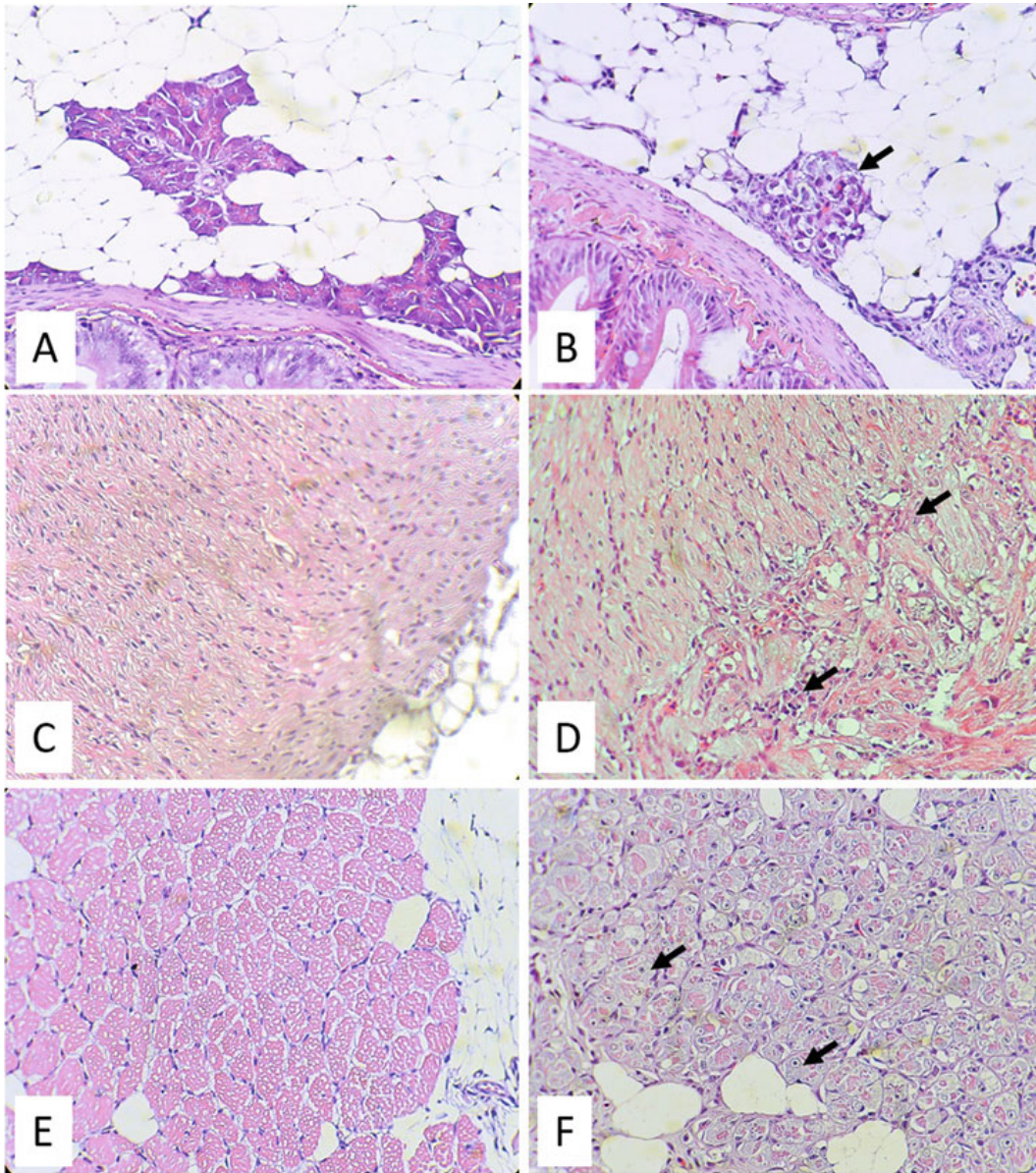
Fig. 4 Demonstration of the histopathology of salmonid alphavirus infection in tissues. The pancreas, heart, and red muscle from health Atlantic salmon (**A**, **C**, **E**) and virus-infected fish (**B**, **D**, **F**). The virus-infected pancreas in (**B**) indicated the total loss of exocrine pancreas, and the inflammatory cells (arrowed) were observed. The virus-infected heart in (**D**) showed the compact and spongy layers in the heart with extensive diffuse myocardiocyte necrosis (arrowed) and the presence of inflammatory cells, with over 50% of myofibers affected. The virus-infected muscle in (**F**) demonstrates the severe diffuse myofibrillar degeneration and inflammatory cells (arrowed)

been classified as "Severe" from virus-infected fish. Each sample should include three sections for blind evaluation and repeated by different trained pathologists to produce results free of observer bias. Besides, for obtaining statistical significance results, the sample size ($n \geq 15$) should be considered.

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<div style="text-align:right">**Chapter 13**</div>

Novel Vaccine Development for Fish Culture Based on the Multiepitope Concept

Sasimanas Unajak, Ansaya Pumchan, Sittiruk Roytrakul, Orathai Sawatdichaikul, and Nontawith Areechon

Abstract

For the past several decades, aquaculture all around the world have been retarded by various disease outbreaks caused by many pathogens including parasites, bacteria, and viruses. Apart from being harmful to human health, the emerging diseases also dramatically affect the farm animals such as livestock and aquatic animals. To cope with this problem, one of the effective prophylactic measures is the application of vaccine. However, the traditional vaccines still have some limitations and several drawbacks; thus there is a need for the development of novel advanced vaccine such as chimeric multiepitope vaccine. Based on the current understanding of genomics and immunoproteomics together with the present bioinformatics tools, the researchers can identify the potential targeted epitopes being recognizable by the immune cells. Additionally, another critical point that should be considered for designing the chimeric multiepitope vaccine is the exposure of all those epitopes to the host organism. Thus, selecting an appropriate linker and joining each identified epitope in a suitable site can create the ideal protein structure protruding all the selected epitopes on its surface. Herein, our study would provide the fundamental platform to develop the multiepitope B-cell vaccine for the prevention and control of the aquatic animal disease starting with the epitope prediction until *in vivo* testing the multiepitope vaccine efficacy.

Key word Chimeric multiepitope vaccine, Reverse vaccinology, Fish vaccine, Subunit vaccine, Streptococcosis, Tim-barrel structure, *In silico* vaccine design, Epitope-based vaccine

1 Introduction

Aquaculture has become one of the most important agricultural activities that supply protein sources all over the world. Due to the superior quality as a protein source, there is a drastic increase in demand for fish for the past several decades. In addition to the declining trend of capture fishery in the open water source of marine and freshwater, the requirements for aquaculture products will be undoubtedly in high demand for global consumers. In 2016, total global fishery products reached 170 million tonnes

(MT), out of which total marine and freshwater aquaculture products were about 80 MT [1]. The average apparent consumption of fishery products in 2016 was 20 kg per capita per year which has been quite stable for the past 5 years. Within aquaculture products, finfish represented about 54 MT followed by crustaceans of about 7.8 MT. For finfish aquaculture, carps and tilapia have been on the top five for recent years counting for about 45%. Tilapia (*Oreochromis niloticus* Linn.) has been the most popular fish, especially in Asia. Annual global production reached over four million metric tons in which about 35% was produced from China and Southeast Asian countries. Tilapia is a hardy species with fast growth and high crop yield with good taste by any cooking means. Most culture techniques have been conducted by an intensive culture system that can yield the optimum harvest of each crop. Unfortunately, this technique has been plagued with many negative consequences especially with deteriorated pond/environmental conditions that weaken the fish in cage or pond. Many etiological agents have been reported especially in the tropical region including parasitic, bacterial, and viral infection. Inevitably, fish farmers must apply chemicals and antimicrobial agents for the control of these infections. However, the treatment response has been usually poor, and many negative consequences have been encountered regarding residual and drug resistance.

Regarding the diseases of Nile tilapia, most of the significant infections have been caused by bacteria. The most common disease is streptococcosis mainly caused by *Streptococcus agalactiae* even though *S. iniae* has also been detected but not as common. Other bacteria are *Aeromonas* spp., *Flavobacterium columnare*, and *Francisella* spp. Recently, the tilapia lake virus (TiLV) has been reported in tilapia farms all around the world but the impact degree has not been that severe compared to bacterial infection. For the sustainable growth of tilapia culture, it is imperative to develop an effective vaccine for the control of *S. agalactiae*. Vaccine development for streptococcosis in tilapia has been continuously investigated since the early 2000 with *S. iniae;* however, recently most of the research was conducted with *S. agalactiae*. Many aspects have been investigated including the serotype profile, vaccine design, and delivery system [2–4]. Some conclusive information has been achieved such as serotype Ia and III of *S. agalactiae* were found to be the main causes of the streptococcosis outbreak in tilapia culture especially in SE Asian regions [5–7]. Vaccine design and delivery systems may be the most challenging issue for the fish vaccine. For finfish, parenteral administration may be the most effective delivery system even though this route can be stressful. Immersion, spraying, and oral administration have also been investigated in many farmed fish including tilapia, but the effectiveness and protection duration are still the main problems of these delivery systems. Regarding vaccine design, the whole-cell inactivated vaccine may be the most common

one due to its simple preparation procedure and safety. Recently, many advanced designs have been investigated including DNA vaccine, subunit vaccine, heterologous live vector vaccine, extracellular products, and so on. The most important factor for the vaccine effectiveness may be the heterogeneity of the target pathogen. Ideally, we need to develop a vaccine that can protect as many serotypes or biotypes as possible within the single vaccine application. Some specific conserved antigenic proteins such as surface immunogenic protein (Sip) may be the most studied protein for the recent investigations in tilapia.

1.1 Subunit Vaccine

Due to the limitation of registered aquaculture vaccines worldwide, the autogenous vaccines, prepared from whole-cell pathogens, maybe the practical approach for vaccine development that can reduce the utilization of antibiotics. However, those registered vaccines might not be effective in some cultivation areas regarding the different strains of pathogens as used in vaccine production. Thus, the variation of pathogen distribution in different cultivation regions may be one important factor that limits the efficacy of many registered vaccines for aquaculture. Moreover, certain types of whole-cell vaccines such as attenuated vaccines, might not be safe for the cultured fish. Therefore, the advantages of subunit vaccines are the design for vaccine effectiveness and appropriateness for upscale production. Importantly, those subunit vaccines do not contain infectious materials. More importantly, subunit vaccines should be the right tool for disease protection for aquatic animals since these vaccines are designed based on either similar or different biomolecules presented in the pathogens to provide broad range or specific protection.

Molecular identification was the first tool used to identify suitable pathogens to be used for rational subunit vaccine design. Later, reverse vaccinology was used to identify antigenic parts of the pathogen using genomics and proteomics analysis, and further used for developing vaccines. The subunit vaccine might be prepared by either single antigen or combined antigens to generate a broad range of vaccines (Fig. 1). However, it is necessary to prove whether those antigenic parts could elicit a protective immune response before efficacy test in animal models.

1.2 Multiepitope Vaccine

Subunit vaccines generated from the single antigenic substance are barely effective when compared with whole-cell vaccine regarding the different number of available antigenic biomolecules. Several multiepitope vaccines were generated to replace the inactivated and attenuated vaccines [8]. In this work, to identify suitable antigenic proteins, comparative immunoproteomics analysis was used to identify these proteins from *S. agalactiae* [8]. Using thorough *in vitro* pull-down assay, an antibody specific to *S. agalactiae* was reacted to *S. agalactiae* proteins. All those proteins were fractionated on 1-dimension electrophoresis (1-DE) and the proteins
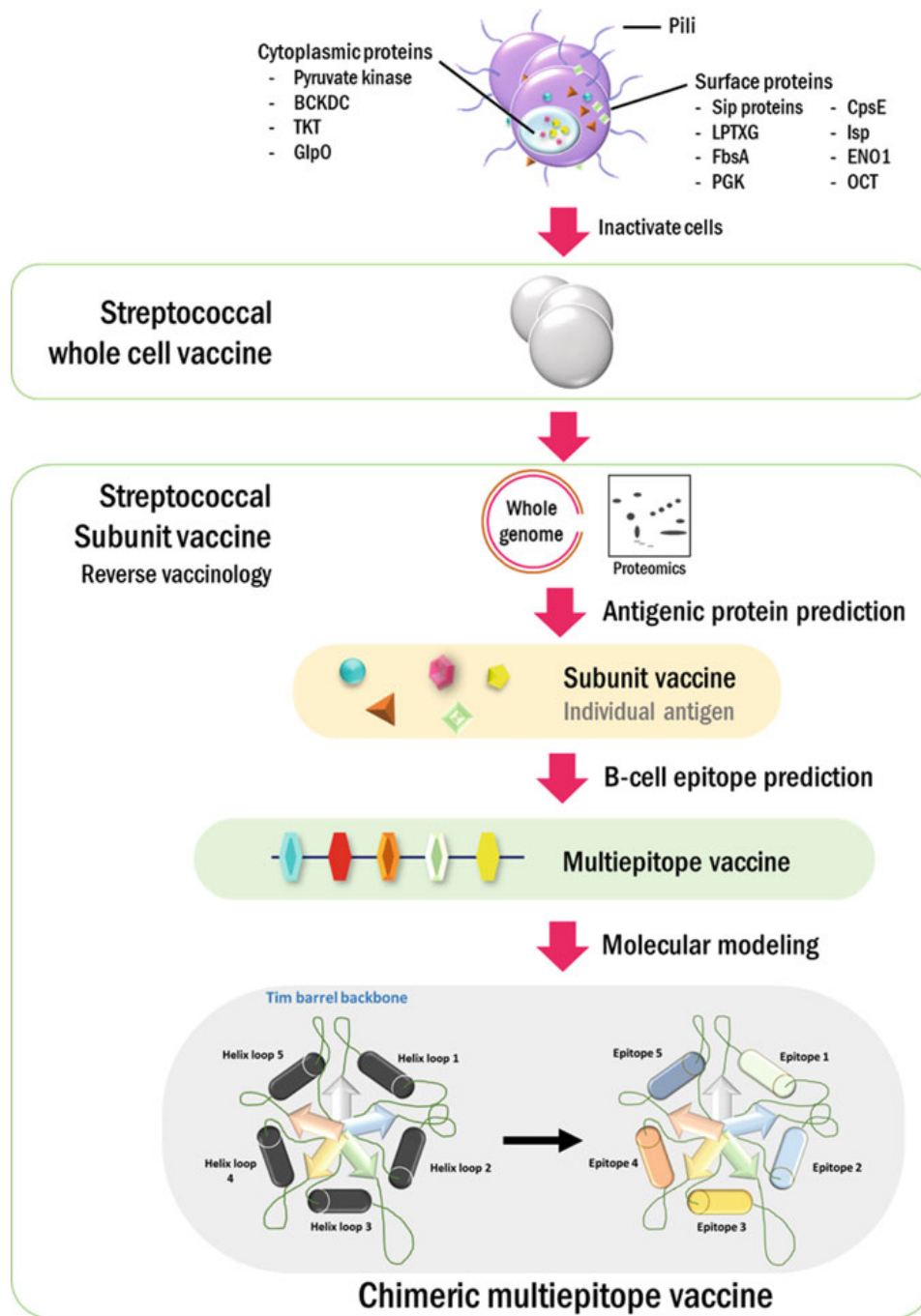
Fig. 1 Development of piscine streptococcosis vaccine. *S. agalactiae* serotype Ia and III isolated from diseased tilapia were used to prepare the vaccine. Whole-cell inactivated vaccine is the first generation vaccine which was prepared by using chemical treated method. Subunit vaccines including DNA and recombinant protein vaccine were prepared from antigenic proteins from *S. agalactiae* serotype Ia and III. Individual antigenic protein could provide sufficient efficacy. However, multiepitope vaccines were generated to provide more variety and the possibility of increase efficacy against different serotypes. Furthermore, the chimeric multi-epitope vaccine was adapted to improve bioavailability which prepared by inserting different epitopes in between the core structure of the Tim-barrel protein backbone

were identified by LC-MS/MS analysis. From these identified antigenic proteins, candidate proteins were subjected to epitope identification. Thus, this epitope identification is the most important step for generating precision multiepitope vaccine design. Apart from that, several bioinformatics tools were used to identify potential epitopes from all those identified antigenic proteins—such as B-cell and T-cell epitopes. However, most of the multimeric vaccines were achieved by joining these epitopes by conjugating with short peptide linker such as EAAAK linker [9–12], GPGPG linker [9–12], AAY linker [9–11], and KK linker [10, 11].

1.3 Concept of Chimeric Multiepitope Vaccine

The concept of multiepitope vaccine design is the providing of the functional and bioavailability of various pathogenic epitopes to host immune system. In this study, to make the chimeric vaccine, flavodoxin protein with Tim-barrel-like structure was used as a backbone and allowed the replacement of different pathogenic epitopes to its α-helices structure (Fig. 2). Thus, the chimeric multiepitope vaccine could design and optimize their tertiary structure to improve the exposure of those epitopes to be recognized by the host immune system, increase the half-life, and retain in the recipient host. Hence, all those antigenic proteins from pathogens, both surface and cytoplasmic proteins, could be used to generate a chimeric multiepitope vaccine.

Chimeric multiepitope vaccines were generated by assembling the antigenic parts of 5 antigenic protein from *S. agalactiae* to the Tim-barrel-like structure of the flavodoxin backbone. The flavodoxin fold has α/β protein topologies. It has three layers, with two α-helical layers sandwiching a 5-stranded parallel β-sheet, while TIM barrel consists of eight α-helices and eight parallel β-strands that alternate along the peptide backbone. Considering these two topologies, TIM barrel is too bulky and complicated to construct the protein structure of chimeric multiepitope. Thus, the flavodoxin fold is fitting to the criteria of vaccine design. The protein structures in databank (RSCB) with flavodoxin fold were screened. The selected flavodoxin fold was utilized as a linker to combine the epitope fragments from five antigenic proteins.

According to Tim-barrel-like structure, there are 5 α-helices and 5 β-pleated sheets in which all those helices are exposed to the surface of the proteins. Therefore, 5 potential epitopes from 5 different antigenic proteins, including C-β protein (BAC), surface protein rib (Rib), LPXTG cell wall anchor domain-containing protein (SPB1), surface immunogenic protein (Sip), and cell surface protein (CSF), were replaced to α-helices of flavodoxin backbone. In silico approaches, including Mathematics and Protein Bioinformatics tools were applied to assist the designation of chimeric multiepitope vaccines. The linker sequences are fixed, whereas the five antigenic peptides are permuted all possibilities of primary structure using an in-house script. Each combination is applied to
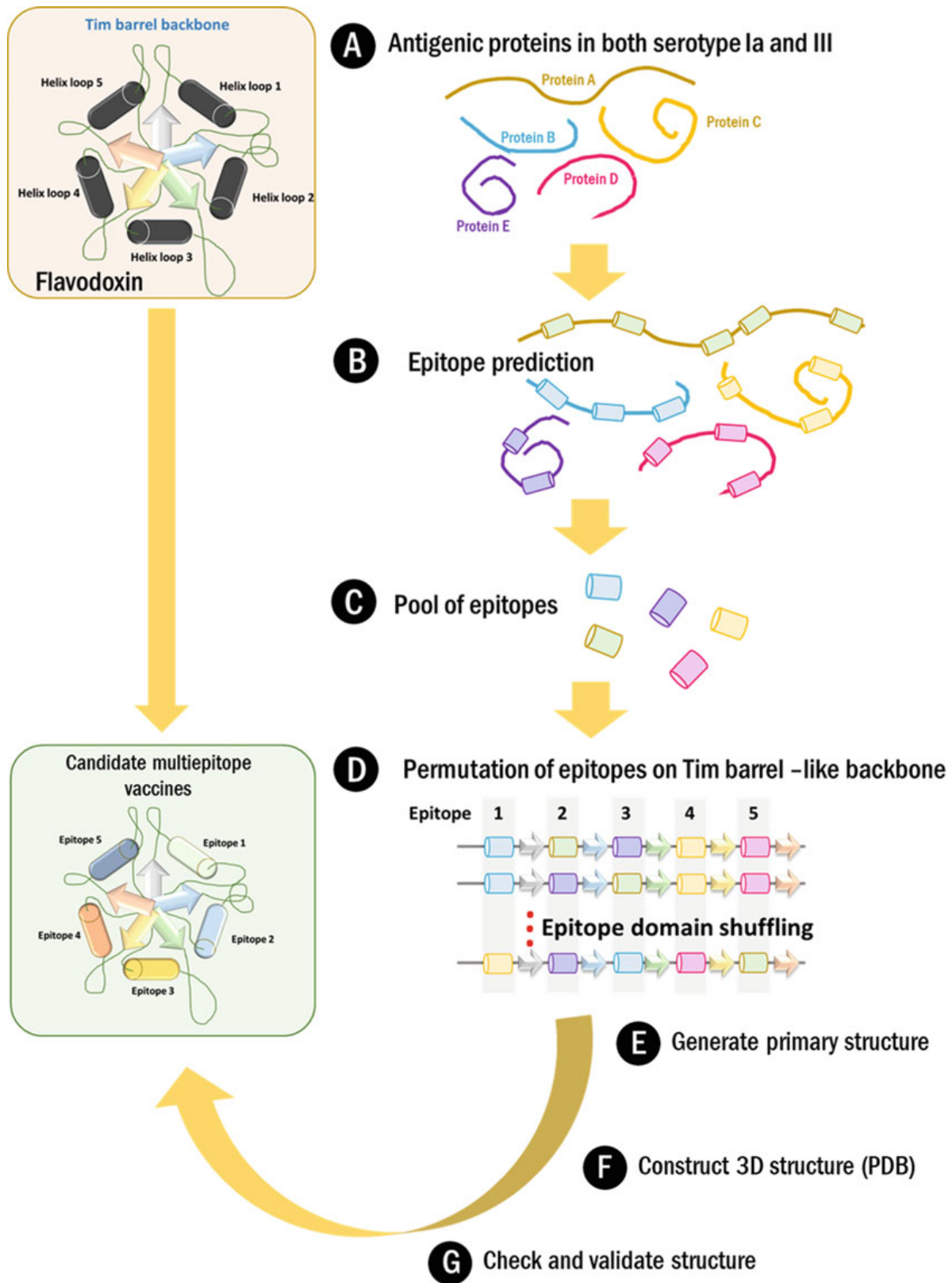
Fig. 2 Working with a multiepitope vaccine. Five different antigenic proteins from *S. agalactiae* were subjected to predict antigenic regions (epitopes) by using BCPRED. The highest score of peptides as predicted by BCPRED was collected and permuted to count all possibilities of five α-helices of Tim-barrel-like structure, flavodoxin fold. After that, all of the structures were determined for characterization and validation of structure

construct primary amino acid sequences and subjected to tertiary structures with "molecular modeling" approaches (Fig. 2). This workflow comes to the precision design of chimeric multiepitope vaccines in which the combinations would produce efficient immunity and effective protection.

2   Materials

2.1   Experimental Fish, Bacterial Strain, Plasmid, Antibody, Antibiotics, and Media

1. Nile tilapia (*O. niloticus*) from GAP farm, Thailand.
2. Tilapia kidney 1 (TK1) cell line (kindly provided by Prof. Ikuo Hirono, Tokyo University of Marine Science and Technology, Japan).
3. *Escherichia coli* DH5α (Invitrogen).
4. *E. coli* Rosetta-gami (DE3) pLysS (Invitrogen).
5. *Streptococcus agalactiae* serotype Ia and III (isolated from diseased tilapia in Thailand [5]).
6. pcDNA3.1(+) vector (Novagen).
7. pET-28a (+) vector (Novagen).
8. pGEM®-T Easy vector (Promega).
9. Anti-his-HRP linked antibody.
10. Anti-mouse IgG HRP-linked antibody.
11. Anti-rabbit IgG HRP-linked antibody.
12. Anti-mouse IgG AP-linked antibody.
13. Anti-rabbit IgG AP-linked antibody.
14. Mouse anti-his antibody.
15. Rabbit anti-flag antibody.
16. Mouse anti-IgM antibody (kindly provided by Assist. Prof. Eakapol Wangkahart, Mahasarakham University, Thailand).
17. *S. agalactiae* serotype III polyclonal antibody (pAb) (kindly provided by Prof. Ikuo Hirono, TUMSAT, Japan).
18. Ampicillin (100 mg/mL).
19. Chloramphenicol (34 mg/mL).
20. Kanamycin (10 mg/mL).
21. Neomycin trisulfate (5–100 mg/mL).
22. Luria-Bertani (LB) medium: 1% tryptone, 0.5% yeast extract powder, 0.5% NaCl.
23. Luria-Bertani (LB) agar plate: 1% tryptone, 0.5% yeast extract powder, 0.5% NaCl, 1.5% Agar.
24. Trypticase Soy agar (TSA): 1% TSA, 1.5% Agar.
25. Trypticase Soy broth (TSB): 1% TSB.

26. Brain Heart Infusion (BHI) agar: 3.7% BHI, 1.5% Agar.

27. 2 × YT medium: 1.6% tryptone, 1% yeast extract powder, 0.5% NaCl.

28. Leibovitz's L-15 completed medium: added 10% FBS, Penicillin–Streptomycin antibiotic for TK1 cell line culture.

2.2 Immunoproteomics Analysis

1. Lysis buffer for lysing *S. agalactiae*: Tris-buffered saline (TBS), 1% Tween-20, 0.01% lysozyme.

2. NuPAGE 4–12% Bis-Tris Protein gel.

3. Protein A agarose beads.

4. Fixing solution: 50% methanol, 10% acetic acid.

5. Coomassie blue R-250 staining solution: 0.1% Coomassie blue R-250, 40% methanol, 10% acetic acid.

6. Destaining solution: 16.5% ethanol, 5% acetic acid.

7. Lysis buffer for gel-free digestion: 0.1% RapidGest SF, 20 mM ammonium bicarbonate.

8. Sequencing-grade trypsin.

9. Mobile phase A: 0.1% formic acid in water.

10. Mobile phase B: 0.1% formic acid in 80% acetonitrile.

11. Ultimate™ 3000 Nano/Capillary LC System (Dionex) coupled with Hybrid quadrupole Q-Tof impact II™ (Bruker Daltonics GmbH) equipped with a Nano-captive spray ion source.

12. Trapping column: PepMap100, C18, 300 μm i.d. × 5 mm (Thermo Scientific).

13. Analytical column: PepSwift C18 Nano Column, 100 μm × 15 cm, i.d. (Thermo Scientific).

14. Bioinformatics software: otofSeries software, CompassXport Version 3.0.9.2, MaxQuant software.

2.3 Chimeric Multiepitope Vaccine Design

1. Bioinformatics software: BCPRED server, I-TASSER server, GalaxyRefine server, PROCHECK program v.3.5.4.

2. In-house script to generate primary sequence permutation (generated by Orathai Sawatdichaikul).

2.4 Codon Optimization

1. Bioinformatics software: OPTIMIZER program (http://genomes.urv.es/ OPTIMIZER /), GeneOptimizer program, RNAfold web server.

2. GeneArt® gene optimization process (Thermo Scientific).

2.5 Chimeric Multiepitope Vaccine Characterization

1. Bioinformatics software: NetNGlyc 1.0 Server, NetOGlyc 4.0 Server, ProtParam server (ExPASy), VaxiJen v2.0 server, ANTIGENpro software, DiscoTope 2.0 server.

2.6 Chimeric Multiepitope Vaccine Preparation

1. Enzymes: Dream *Taq* DNA polymerase (Thermo Scientific), Ex *Taq* DNA polymerase (Takara), T4 DNA ligase (Thermo Scientific), *EcoR*I (Thermo Scientific), *Nru*I (Takara), *BamH*I (Thermo Scientific), *Xho*I (Thermo Scientific).

2. Kits: Effectene Transfection Reagent (Qiagen), GeneJET Gel Extraction (Thermo Scientific), GeneJET Plasmid Miniprep (Thermo Scientific), SuperSignal™ West HisProbe™ Kit (Thermo Scientific), Immobilon®Forte Western HRP Substrate (Millipore).

3. Synthesized chimeric multiepitope vaccine in pMA_T vector.

4. Primers for multiplex PCR to identify serotype of *S. agalactiae* [5] (5′→3′oligonucleotide sequences):
   cpsI-Ia-6-7 Fw (GAATTGATAACTTTTGTGGATTGC GATGA),

   cpsL Fw (CAATCCTAAGTATTTTCGGTTCATT),

   cpsL Rv (TAGGAACATGTTCATTAACATAGC),

   cpsG Fw (ACATGAACAGCAGTTCAACCGT),

   cpsG Rv (ATGCTCTCCAAACTGTTCTTGT),

   cpsG-2-3-6 Rv (TCCATCTACATCTTCAATCCAAGC),

   cpsJ-Ib Fw (GCAATTCTTAACAGAATATTCAGTTG),

   cpsJ-Ib Rv (GCGTTTCTTTATCACATACTCTTG).

5. Primers for checking plasmid insertion in target vector:
   BGH Rv (TAGAAGGCACAGTCGAGG),

   T7 Promoter (TAATACGACTCACTATAGGG),

   T7 Terminator (GCTAGTTATTGCTCAGCGG).

6. Isopropyl β-D-1-thiogalactopyranoside (IPTG): 0.1 mM IPTG in isopropanol.

7. Lysis buffer: 50 mM Tris–HCl (pH 7.4), 1 mM PMSF.

8. Tris-Glycine buffer: 0.2 M glycine, 0.025 M Tris–HCl, 3.47 mM sodium lauryl sulfate.

9. 12% SDS–PAGE.

10. 4× SDS loading dye: 0.2 M Tris–HCl (pH 6.8), 0.4 M DTT, 277 mM SDS, 6 mM Bromophenol blue, 4.3 M glycerol.

11. Coomassie blue R-250 staining solution: 10% acetic acid, 50% methanol, 0.1% Coomassie blue R-250.

12. Destaining solution: 10% acetic acid, 50% methanol.

13. 1× TBST buffer: 1× TBS, 0.1% Tween 20.

14. Blocking solution: 0.1% BSA in 1 × TBST buffer.

15. Ni-NTA agarose bead (Qiagen).

16. $1 \times$ PBS buffer (pH 7.4): 0.2 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4.

17. Elution buffer: 5–500 mM imidazole, $1 \times$ PBS buffer (pH 7.4).

18. NBT/BCIP substrate (Sigma-Aldrich).

19. Solution I: 50 mM D-glucose, 25 mM Tris-pH 8, 10 mM EDTA.

20. Solution II: 2% SDS, 0.4 M NaOH.

21. Solution III: 3 M CH_3COOK, 5 M CH_3COOH.

22. $1 \times$ TE buffer (pH 8).

23. RNase A (4 mg/mL).

24. Ethidium bromide (EtBr) (10 mg/mL).

25. Serva DNA stain G (SERVA).

26. 25 cm^2 T flasks for fish cell culture.

27. NanoDrop One spectrophotometer.

28. T100 ™ Thermal Cycler.

29. Submarine gel electrophoresis.

30. Gel Doc ImageQuant LAS 500.

31. High-speed Refrigerated microcentrifuge.

32. Fast protein liquid chromatography (FPLC) *ÄKTA™ FPLC* P900 incorporated with a HiPrep 16/60&26/60 Sephacryl S-300 High-Resolution column.

33. PVDF membrane.

34. Blue light transilluminators.

35. Trans-Blot Turbo Transfer System.

36. SDS-PAGE electrophoresis apparatus.

37. Ultrasonic Processor.

38. Ultracentrifuge.

39. Vivaspin® 20, 10 kDa MWCO.

2.7 Vaccine Efficacy Analysis

1. Montanide ISA 763 (Seppic).

2. Formalin-killed cells (FKC) vaccine of *S. agalactiae* serotype III in 1×10^8 CFU/mL as the positive control.

3. *S. agalactiae* serotype III (1×10^7 CFU/mL) for a challenge test.

4. ChemiDoc™ Imaging System (Bio-Rad).

5. ImageJ program version 1.x.

6. Minifold® I dot blot system.

3   Methods

3.1 Immunoproteomics Analysis

3.1.1 Immunogenic Protein Precipitation

1. *S. agalactiae* serotype Ia and III were cultured in BHI broth at 30 °C until the exponential phase was reached.

2. Harvest bacterial cell by centrifugation at $10,000 \times g$ for 10 min at 4 °C.

3. Wash pellet with chilled $1 \times$ PBS, resuspend in 100 μL lysis buffer and incubate at 50 °C for 20 min.

4. Incubate at -20 °C for 30 min, sonicate on ice until the solution became clear.

5. Collect supernatant after centrifugation at $10,000 \times g$ for 20 min followed by protein concentration analysis by Lowry assay.

6. Add 500 μL of 1 μg/μL of protein A agarose bead to bacterial protein lysate, and centrifuge at $10,000 \times g$ at 4 °C for 10 min to remove nonspecific proteins.

7. Add 5% glycerol to the clarified supernatant following with pAb specific to *S. agalactiae* serotype III (1:500 dilution) and then incubate at 4 °C for 16 h.

8. Put 30 μL of protein A agarose beads and incubate at 4 °C for 3 h to separate bound immunogenic proteins.

9. Purify the bound proteins by acetone precipitation [1:5 (v/v)].

10. Solubilize precipitated proteins in 20 mM Tris–HCl, pH 8.0 with 0.5% SDS followed by protein concentration analysis using Lowry assay.

11. Assess protein profile by fractionating 25 μg of protein on a NuPAGE 4–12% Bis-Tris Protein gel.

12. Overlay the gel in fixing solution for 3 h, stain with Coomassie blue R-250 staining solution, and eliminate an excess blue background with destaining solution.

3.1.2 Gel-Free Digestion for Immune-Proteomics Approach

1. Mix 3 μg of immunogenic protein with lysis buffer for gel-free digestion and incubate with 5 mM DTT in 10 mM ammonium bicarbonate (NH_4HCO_3) at 60 °C for 3 h to reduce sulfhydryl bonds.

2. Incubate with 15 mM IAA (2-Iodoacetamide) in 10 mM NH_4HCO_3 at 25 °C for 45 min in dark for alkylation of sulfhydryl groups.

3. Clean up the protein solution by Zeba Spin Desalting Column before incubation with 50 ng of sequencing-grade trypsin at 37 °C for 6 h.

4. Dry at 44 °C under a vacuum and protonate with 0.1% formic acid in LC-water before injection into an LC-MS/MS.

3.1.3 Immunogenic Proteins Identification by LC-MS/MS

1. Subject 500 nL of extracted peptide to a trapping column through a full loop injection before being resolved in an analytical column at 60 °C.

2. Elute peptides with mobile phase A and mobile phase B at a 0.35 μL/min constant flow rate into the mass spectrometer and then conduct with electrospray ionization with Captive-Spray at 1.6 kV (*see* **Note 1**).

3. Collect raw LC-MS/MS spectra using CompassXport to convert all spectra into the mzXML data format.

4. Evaluate the mzXML files for label-free quantification of peptides based on the MS profile by Maxquant software (*see* **Note 2**).

3.2 Chimeric Multiepitope Vaccine Design

The bioinformatics framework designing chimeric multiepitope vaccine is shown in Fig. 3.

1. Predict linear B-cell epitope of the sharing immunogenic proteins (from *S. agalactiae* serotype Ia and III) and previously classified virulence proteins using BCPRED server.

2. Choose the identified epitopes with providing the highest BCPRED score.

3. Screen flavodoxin fold in RSCB server to use as flavodoxin linker (PDB accession code: 3CHY) *in silico* (*see* **Note 3**).

4. *Permute* all possibilities of selected epitopes using the in-house script.

5. Assembly each *permutation* with the flavodoxin linker and generate primary structure/sequence.

6. Construct a 3D structure by I-TASSER server based on an alignment of multiple threading templates using qualifying C-score value.

7. Select a repeated structure perturbation and the best structural relaxation candidates, and then convert to PDB file using the GalaxyRefine server. Determine amino acid residues' stereochemical quality for all the refined chimeric multiepitope models by the PROCHECK program and validate the best models through Ramachandran plots (*see* **Note 4**).

3.3 Codon Optimization

1. After obtaining the appropriate chimeric multiepitope vaccine design, reverse-translate amino acid sequences to nucleotide sequences using Nile tilapia codon usage (*Oreochromis niloticus* [gbvrt]: 113) from Kazusa codon usage database.

2. Analyze codon adaptation index (CAI) of nucleotides by optimizer program combined with GeneArt® Gene Synthesis (Thermo Fisher Scientific) (*see* **Note 5**).
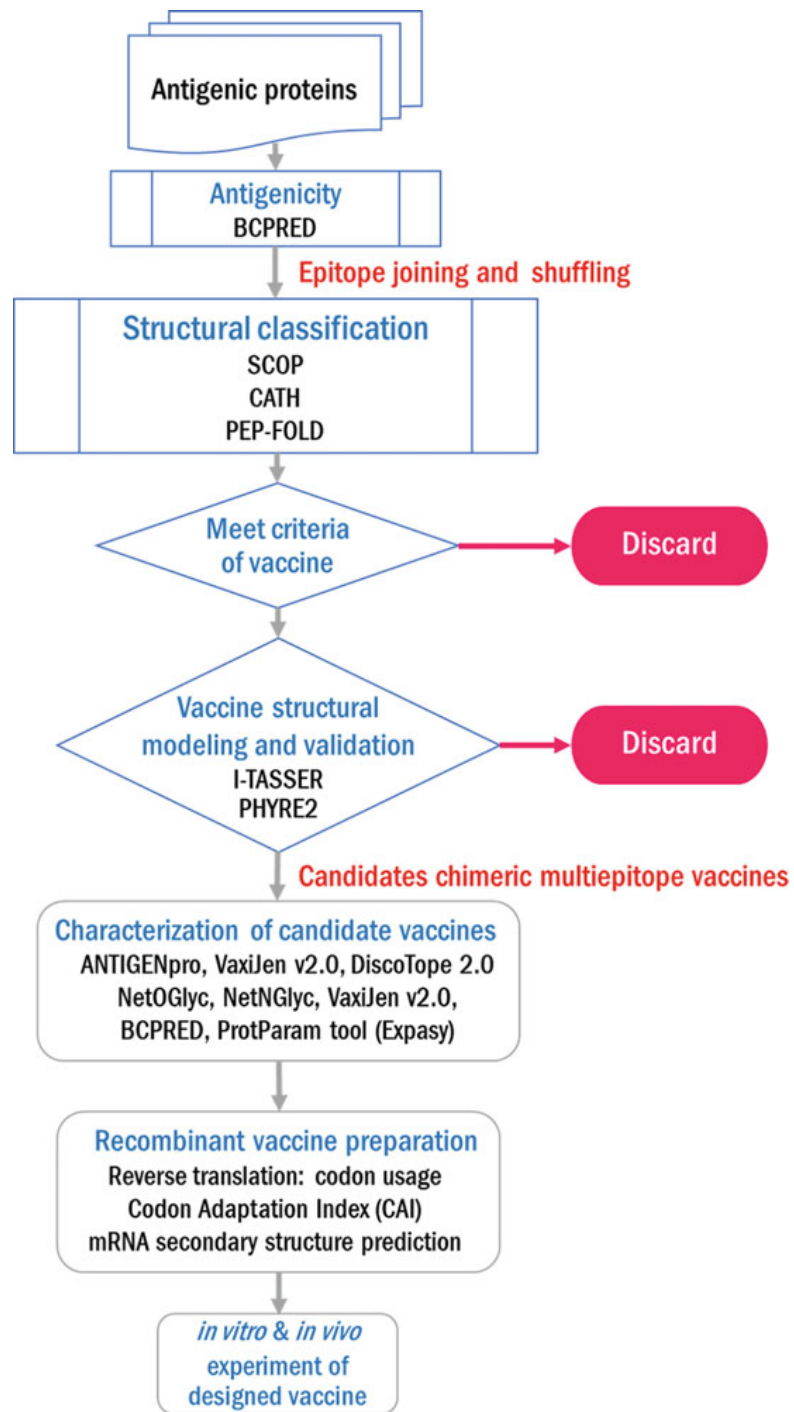
Fig. 3 Pipeline in silico development of chimeric multiepitope vaccines. Different bioinformatics programs were used to prepare epitopes for aligning them on the core structure of Tim-barrel-like structure. The best candidate chimeric multi-epitope vaccine as determining by I-TASSER and PHYRE2 has further validated their biochemical properties by several bioinformatics programs

3. Evaluate the secondary structure of the single-stranded RNA folding and free energy of the thermodynamic ensemble by RNAfold web server.

4. Before nucleotide synthesis, add the start codon and stop codon at 5′ end and 3′end, put double restriction sites of *BamH*I and *Xho*I to the 5′ end and 3′end that corresponded with pcDNA3.1(+) and pET28a(+) vectors, also add Flag tag (DYKDDDDK) at 3′end for detecting the chimeric multiepitope protein expression and further purification.

5. Synthesize the optimized DNA sequence by GeneArt® Gene Synthesis.

3.4 Chimeric Multiepitope Vaccine Characterization

1. The completed sequences of the chimeric multiepitope vaccine were evaluated for molecular characterization by computational methods.

2. Examine *N*-linked and *O*-linked glycosylation sites using NetNGlyc 1.0 Server and NetOGlyc 4.0 Server.

3. Predict the theoretical pI (isoelectric point), MW (molecular weight), the composition of positive and negative residues, estimated half-life, extinction coefficient, aliphatic index, and grand average of hydropathicity (GRAVY) through the Prot-Param server of ExPASy.

4. Analyze antigenicity with the VaxiJen v2.0 server and ANTI-GENpro software.

5. Define discontinuous B-cell epitopes by the DiscoTope 2.0 server (use PDB format) at the default threshold of -1.0 to -3.7.

3.5 Chimeric Multiepitope Vaccine Preparation

To prepare the chimeric multiepitope vaccine, the multiepitope fragments was synthesized in commercial pMA_T plasmid. The synthesized multiepitope gene was double digested with specific restriction enzymes and put in both bacteria and eukaryotic expression vectors. Those recombinant plasmids harboring the multiepitope gene were constructed and transferred to *E. coli* (Rosetta) and fish cell line (TK-1 cells), respectively. The expression of chimeric multiepitope protein expression in bacteria and eukaryotic expression were verified and prior efficacy tested in the fish model. Importantly, with this multiepitope production platform, not only recombinant multiepitope vaccine was achieved, but pcDNA plasmid harboring the multiepitope gene was also used as a DNA vaccine and provided similar protection. The pipeline of chimeric multiepitope vaccine preparation and verification are shown in Fig. 4.
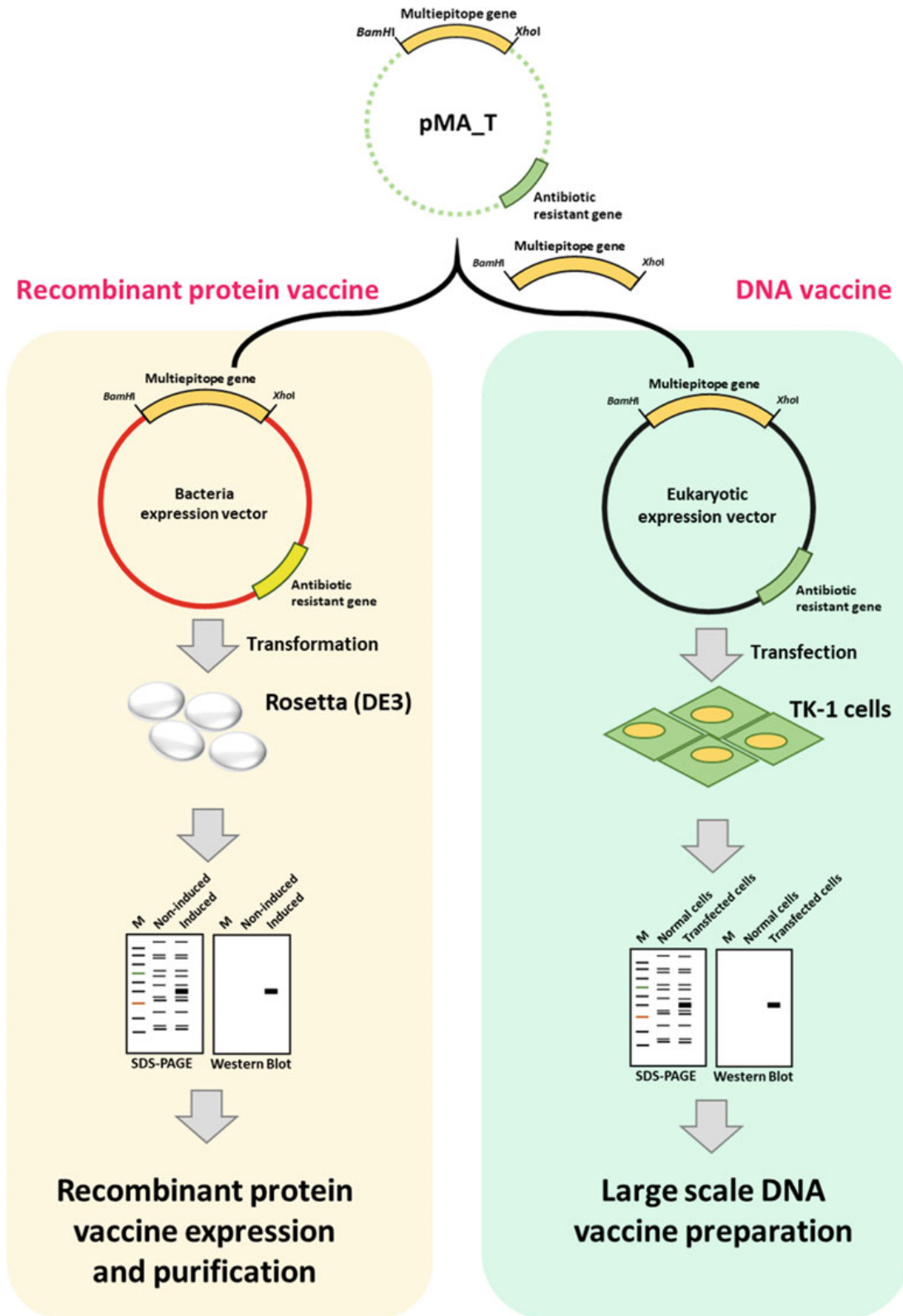
Fig. 4 Synthesized chimeric multiepitope vaccine was digested from pMA_T plasmid using restriction enzymes. The DNA fragment was put into two different plasmids to prepare two different systems of subunit vaccine: recombinant proteins (in bacterial expression system) and DNA vaccine (in eukaryote expression system). Achievement of recombinant protein expression was tested in *E. coli* and fish cell lines before testing vaccine efficacy in Nile tilapia (*Oreochromis niloticus*)

3.5.1 Construction of Plasmid Harboring Chimeric Multiepitope Vaccine

1. Transform the pMA_T vector harboring synthesized chimeric multiepitope gene into *E. coli* (DH5α) through heat shock method for multiplication.

2. Cut the synthesized gene out of the vector by double restriction enzymes, *Bam*HI and *Xho*I, run on 1% agarose gel and then purify DNA fragment by GeneJET Gel Extraction kit.

3. Sequence DNA sequencing (Macrogen) before inserting the fragment into the expression plasmid as pET28a (+) and pcDNA3.1(+).

4. Determine an accomplished insertion through PCR with T7 promoter and T7 terminator primers for pET28a(+) vector and with T7 promoter and BGH Rv primers for pcDNA3.1(+) vector.

5. Analyze the DNA fragments using electrophoresis 1% agarose gel containing Serva DNA stain G and visualize by Gel documentation system.

6. Extract plasmid of pET28a (+) and pcDNA3.1(+) harboring the synthesized gene from *E. coli* (DH5α) by GeneJET Plasmid Miniprep kit following manufacturers protocol.

3.5.2 Chimeric Multiepitope Protein Vaccine Expression in the Prokaryotic System

1. Verify the prokaryotic expression system by transformation of the pET28a(+) vector harboring chimeric multiepitope vaccine into *E. coli* Rosetta-gami (DE3) pLysS strains using the heat shock method.

2. Induce protein expression in LB broth containing 10 mg/mL kanamycin and 34 mg/mL chloramphenicol at 30 °C for 3 h with 0.1 mM IPTG.

3. Collect bacterial cells, resuspend in lysis buffer, sonicate under 20% amplitude with a pulse on/off at 5 s/5 s condition, and centrifuge under 4 °C at $8000 \times g$ for 15 min to separate the pellet and supernatant.

4. Prepare protein samples including whole cell, pellet, and supernatant of the induced and non-induced bacterial cell by adding 4x loading dye before heating at 95 °C for 20 min, and then perform 12% SDS-PAGE.

5. Separate one gel for staining with Coomassie staining solution and another for western blot analysis.

6. Conduct western blot analysis by transferring protein bands from SDS-gel to PVDF membrane using Trans-Blot Turbo Transfer System, soak the membrane in blocking solution for 1 h, wash with $1 \times$ TBST three times for 5 min, and incubate with anti-his-HRP linked antibody for 1 h (1:10000).

7. Wash thrice with $1 \times$ TBST, incubate with a substrate of SuperSignal™ West HisProbe™ for 5 min in darkness, and visualize by Gel Doc system in the chemiluminescent mode.

3.5.3 Chimeric Multiepitope DNA Vaccine Expression in the Eukaryotic System

1. Verify the ectopic expression of the chimeric multiepitope DNA vaccine by transfection of the pcDNA3.1(+) vector harboring the synthesized gene into TK1 (Tilapia Kidney 1) tilapia cells using Effectene Transfection Reagent as the kit's guideline protocol.

2. Maintain the transfected fish cell cultures with Leibovitz's L-15 completed medium in 25 cm^2 T-flask.

3. After 1 week-post-transfection, collect the fish cells to monitor chimeric multiepitope DNA vaccine expression by SDS-PAGE and western blot analysis.

4. Markedly, use rabbit anti-flag antibody (1:10,000) and anti-rabbit IgG AP-linked antibody (1:10,000) as primary antibody and secondary antibody followed by developing the signal with NBT/BCIP substrate.

3.5.4 Chimeric Multiepitope Recombinant Protein Vaccine Purification

1. Culture *E. coli* Rosetta-gami (DE3) pLysS containing the pET28a (+) vector harboring chimeric multiepitope vaccine under the previously mentioned conditions in large scale.

2. Purify the expressed protein by Ni-NTA agarose bead column with a gradient concentration of imidazole ranging from 5 mM to 500 mM in $1 \times$ PBS.

3. Investigate the quality of the purified protein by 12% SDS-PAGE analysis.

4. Pool the fractions containing target protein together and concentrate with the Vivaspin 10 MWCO centrifugal concentrator.

5. Subsequently, purify the target protein by the gel filtration chromatography method through Fast Protein Liquid Chromatography (FPLC) incorporated with HiPrep 16/60&26/60 Sephacryl S-300 High-Resolution column using $1 \times$ PBS buffer with 1 mL/min flow rate to elute the protein out depending on its retention time.

6. Confirm the purity of chimeric multiepitope protein vaccine by 12% SDS-PAGE analysis and western blot analysis using anti-his-HRP linked antibody (1:10,000).

7. Measure the protein concentration and keep in -20 °C until use.

3.5.5 Chimeric Multiepitope DNA Vaccine Preparation

1. Conduct DNA plasmid purification by ultracentrifugation using the CsCl gradient method.

2. Firstly, inoculate *E. coli* DH5α strain containing the pcDNA3.1 (+) vector harboring chimeric multiepitope DNA vaccine in 3 mL of LB broth with 10 mg/mL kanamycin at 37 °C for 7–9 h approximately.

3. Transfer half of the pre-culture to 200 mL 2 × YT broth before culture at 37 °C overnight and move to a centrifuge tube to collect the pellet by centrifugation with 5000 × *g*, 4 °C for 10 min.

4. Resuspend the pellet with 5 mL Solution I before adding and strongly mixing with 5 mL of Solution II.

5. Add 7.5 mL Solution III to the mixture and incubate on ice for 5 min following centrifugation under 4 °C at 9000 × *g* for 15 min.

6. Transfer the supernatant to 50 mL tube, add isopropanol (0.6 × amount of supernatant), and incubate at −20 °C for 30 min to precipitate plasmid DNA.

7. Discard supernatant after centrifuging at 4 °C, 6000 × *g* for 20 min and wash in 3 mL of 75% ethanol.

8. Collect the pellet of plasmid DNA by centrifugation at 7000 × *g* for 10 min at 4 °C, and dry at room temperature before dissolving in 3 mL 1 × TE buffer.

9. Add 50 μL RNase A solution, incubate at 37 °C for 1 h, add CsCl 3.88 g/tube and mix thoroughly.

10. Put 150 μL of EtBr, then add 1 × TE buffer until full and mix carefully to avoid bubbles.

11. Detect the pink color band of the plasmid DNA vaccine under UV light after centrifugation using the ultracentrifuge at 22 °C and 65,000 × *g* for 18 h.

12. Extract DNA sample by a 1 mL syringe injector and put to 1.5 mL tubes.

13. Add saturated isopropanol (same amount of the samples) and centrifugate at 13000 × *g*, 4 °C for 10 min to remove pink color supernatant (repeat until the pink color disappear).

14. Dialyze the derived plasmid of DNA vaccine using 1 × TE buffer by conducting the first dialysis for 4 h and the second overnight at 4 °C.

15. Measure the concentration of the dialyzed chimeric multiepitope DNA vaccine through Nanodrop spectrophotometer and analyze DNA integrity via electrophoresis before keeping in −20 °C for further vaccine efficacy analysis.

3.6 Vaccine Efficacy Analysis

3.6.1 Formalin-Killed Vaccine Preparation

1. Culture *S. agalactiae* bacteria (serotype Ia and III) in 5 mL of BHI broth as the starter and incubate at 30 °C overnight with 220 rpm shaking.

2. Add the starter culture into 100 mL of BHI (1:5000) before shaking at 220 rpm and 30 °C for approximately 20 h.

3. Collect cell pellet in 50 mL tube by centrifuging at $4000 \times g$ in 25 °C for 10 min.

4. Wash with 0.85% NaCl solution and centrifuge at $4000 \times g$ in 25 °C for 10 min.

5. After discarding the supernatant, repeat the washing step twice.

6. Soak the bacterial pellet with 0.85% NaCl containing 1% formaldehyde before keeping in 4 °C overnight.

7. Wash out formalin from bacterial suspension by centrifugation twice and resuspended in 0.85% NaCl.

8. Take 100 μL of the mixture, and then spread on BHI agar to check that all bacteria are killed. If there was bacterial growth, then repeat the **steps 6–7**.

9. Collect the pellet by centrifugation and wash three times with 0.85% NaCl solution.

10. Soak and resuspend the pellet with 0.85% NaCl containing 0.1% formaldehyde.

11. Keep the vaccine in 0.1% formaldehyde in 4 °C until used.

3.6.2 Fish Vaccination

1. To evaluate vaccine efficacy, Nile tilapia (*O. niloticus*) were immunized with chimeric multiepitope vaccines (recombinant protein and DNA vaccines), followed by bacterial challenge.

2. Divide the experimental fish to 4 groups, namely, (1) the recombinant protein vaccine, (2) DNA vaccine, (3) formalin—killed (FKC) *S. agalactiae* vaccine, and (4) pcDNA3.1(+) [empty vector] in triplicate.

3. Acclimatize 25 disease-free Nile tilapia (60 ± 5 g) into 12 glass aquarium tanks containing 30 L of water for one week before vaccination.

4. Vaccinate the purified protein vaccine mixed with Montanide adjuvant (in 7:3 ratio) through intraperitoneal (IP) injection at 200 μg (in 100 μL) of protein per fish for the chimeric multiepitope protein group, and inject 10 μg of the purified plasmid of DNA vaccine through intramuscular (IM) route to the fish for the chimeric multiepitope DNA vaccine.

5. Use FKC mixed Montanide as the positive control, and pcDNA3.1(+) as the negative control.

6. During 4 weeks of post-vaccination, collect blood from the fish caudal vein of 5 fish in each treatment once a week to obtain

serum for immunoblotting assay, and transfer those fish to another separate tank to avoid contamination.

7. Maintain all fish under running and aerated water at $30 \pm 3\,°C$, and feed with commercial pellet feed twice a day.

3.6.3 Challenge Test

1. On the fifth week, take 30 vaccinated fish (10 per replicate) of each experimental group from the remaining fish and anesthetize with eugenol before injecting with *S. agalactiae* (serotype III) at 1×10^7 CFU/mL through IP administration.

2. Record mortality and clinical signs of infected tilapia daily for three weeks.

3. Collect brain, head kidney, and liver from moribund fish to isolate the bacteria on BHI agar, and confirm the serotype of *S. agalactiae* by multiplex PCR method using eight primers under $95\,°C$ for 5 min following 15 cycles of $95\,°C$ 1 min, $54\,°C$ 1 min and $72\,°C$ 1 min before performing 25 cycles of $95\,°C$ 1 min, $56\,°C$ 1 min and $72\,°C$ 2 min; and $72\,°C$ 10 min condition.

4. Calculate cumulative mortality and relative percentage survival (RPS) {RPS = [1 − (% fish mortality of vaccinated/% fish mortality of fish control)] × 100}.

5. Statistical analysis to consider the significant difference between the treatment by one-way analysis of variance (ANOVA) and multiple comparisons by Duncan's New Multiple Range Test. Differences were considered significant at $P < 0.05$.

3.6.4 Immunoblotting Assay

1. To detect the antibody response after immunization, antibody production was evaluated through dot blot analysis using the Minifold® I dot blot system.

2. Briefly, activate the PVDF membrane with methanol and wash with transfer buffer before being placed on 3 blotting papers.

3. Spot 20 μL of purified chimeric multiepitope protein (10 μg/mL) on the membrane and then incubate with blocking solution for 20 min.

4. After washing with TBST buffer by pipetting, add 10 μL of serum of the different treatment groups as stated above, and then take the membrane out and probe with Mouse anti-IgM antibody (1:5000) for 1.5 h.

5. Wash three times with TBST buffer and incubate in Anti-mouse IgG HRP-linked antibody solution (1:10000) for 45 min.

6. Subsequently, detect the signal with ChemiDoc™ Imaging System after adding the Forte western HRP Substrate.

7. Interpret the integrated density of the dot blot by ImageJ before presenting as a bar chart with statistical analysis.

4 Notes

1. The Mass spectra (MS) and MS/MS spectra were fully acquired in positive ion mode (Compass 1.9 for OTOF series software,). Mass accuracy was assessed using positive detection mode after internal calibration with sodium trifluoroacetate (Na-TFA) with 1.6 ppm. Briefly, LC-MS/MS spectra were acquired using a data-dependent auto-MS/MS method as a dynamic method with a fixed cycle time of 3 s. Mass spectral information was collected +2, +3, and +4 charge states with m/z range from 400 to 2200.

2. MaxQuant 1.6.1.12 was used to quantify the proteins in individual samples using the Andromeda search engine to correlate MS/MS spectra to the Next-generation sequencing database. The following parameters were utilized for data processing: maximum of Three miss cleavages, first search peptide tolerance of 0.07 Da, main search peptide tolerance of 0.006 Da, intensity threshold of 30, trypsin as digesting enzyme including carbamidomethylation of cysteine as a fixed modification, and the oxidation of methionine and acetylation of the protein N-terminus as variable modifications. Only peptides with a minimum of 7 amino acids, as well as at least one unique peptide, would be required for protein identification. Only proteins with at least two peptides and at least one unique peptide were considered as being identified and used for further data analysis.

3. Based on protein fold classes, generally recognized classes of protein, namely, all-α, all-β, α + β and α/β that were usually considered in the two main structure classification databases. SCOP and CATH databases, were introduced to design an appropriate chimeric multiepitope vaccine structure. Herein, the structural domain of the α/β class would be focused due to performing α-helices and parallel-β-strands alternatively throughout the backbone probably providing potential bioactivity. Among α/β proteins, the flavodoxin fold type of CheY from *Escherichia coli* was utilized to be the suitable linkers combining epitope fragments of five antigenic genes. According to its native construct 5 α-helices protrude out of the structure, therefore, all α-helix chosen epitopes could be fitted to this flavodoxin fold.

4. PROCHECK program generated the Ramachandran plots based on the calculation of phi-psi torsion angles of each amino acid residue. The stereochemical quality of protein

structures would be acceptable if the amino acid residues located in most favored regions as much as possible and fell in disallowed regions less than 2%.

5. The ectopic expression of bacterial protein in the fish cells might not be achieved due to different codon utilization in the bacterial system. Subsequently, codon optimization of the chimeric multiepitope vaccine should be analyzed by GeneArt™'s gene optimization according to ISO 9001 standards (registration no. 1210024212) to apply the codon bias of *Oreochromis niloticus*. The region of an ideal GC content range—between 30% and 70%—was well optimized. Moreover, negative cis-acting sites included internal TATA-boxes, chi-sites, and ribosomal sites; AT-rich or GC-rich sequence stretches; RNA instability motifs; repeat sequences; RNA secondary structures; and splice donor and acceptor sites in higher eukaryotes, which were successfully removed from these chimeric multiepitope DNA vaccine sequences.

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Chapter 14

Testing Novel Inactivation Methods and Adjuvants for Vaccines Against *Streptococcus agalactiae* in Nile Tilapia *Oreochromis niloticus*

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Abstract

Inactivation by hydrogen peroxide and pH manipulation are two novel methods used recently in experimental vaccines against *Streptococcus agalactiae* in Nile tilapia. Here we describe in detail inactivation using novel methods as well as the classical method of inactivation. These vaccines showed similar moderate efficacy when compared to the conventional formaldehyde vaccine. In addition, we describe the inclusion of adjuvants in a hydrogen peroxide vaccine.

Key words Streptococcosis, Hydrogen peroxide, Bacterial inactivation, Aluminum hydroxide, Vaccination

1   Introduction

Streptococcus agalactiae, a group B streptococcus, is a Gram-positive bacterium [1], which is responsible for high morbidity and mortality in freshwater, estuarine and marine fish, including Nile tilapia [2] and has been reported to cause septicemia and meningoencephalitis [1]. Outbreaks of this disease have been reported worldwide [3]. Streptococcosis is widely distributed in several states of Brazil [4]. Vaccination is an effective method to control *S. agalactiae* infection and prevent mass mortality in tilapia [5]. Currently, most vaccines available to fish farmers are inactivated, as they are easier and cheaper to produce and ecologically safer than live vaccines [6]. Most vaccines use formaldehyde as inactivating agent in fish vaccines [7–9]. Nevertheless, formaldehyde could alter the physical and chemical characteristics of

superficial antigens [10, 11]. Hence, alternative inactivation methods such as *h*ydrogen peroxide (H_2O_2) have been tested for inactivation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in animal models of vaccines [12], reporting that H_2O_2 is less toxic and preserve epitopes better when compared to the classical method by formaldehyde. Also, this inactivation method showed high efficacy and improvement of the adaptive immune response when tested in rhesus monkeys and mice against West Nile virus [12–14]. Bacterial inactivation methods based on pH manipulation have also been tested for elaboration of vaccines against *Yersinia ruckeri* in Atlantic salmon, *Salmo salar* [15]. Recently, our research group compared the efficacy of $H_2O_{2\_}$inactivated and pH-manipulated vaccine against *S. agalactiae* in Nile tilapia in relation to formaldehyde-inactivated vaccine [16]. In the present chapter, we describe in detail two novel inactivation methods and a classical method (H_2O_2-inactivation, inactivation by pH inactivation and formaldehyde-inactivation), which were published in a comparative study about novel inactivation methods for a vaccine against *Streptococcus agalactiae* in Nile tilapia [16]. In addition, we describe in detail the indirect enzyme linked immunosorbent assay test for determination of anti-*S. agalactiae* IgM antibodies and methods to prepare two adjuvants added to a novel $H_2O_{2\_}$inactivated vaccine [17].

2   Materials

2.1   Bacterial Strain

Pathogenic strain of *Streptococcus agalactiae* (serotype Ib, strain SA43) isolated from Nile tilapia with clinical signs of streptococcosis in the northern region of Paraná State, Brazil [18]. Biochemical identification and serological classification were performed according to Salvador et al. [19] and species identity of the isolates was confirmed by polymerase chain reaction (PCR) amplification of the 16S/23S rRNA intergenic spacer region [20].

2.2   Growth Media

1. Brain Heart Infusion Agar (BHI) (Kasvi, Brazil): 52 g dissolved in 1 L of distilled water. Autoclave for 15 min at 121 °C. Composition (g/L): 10 g beef brain heart infusion, 10 g peptone, 7.5 g calf brain heart infusion, 5 g sodium chloride, 2.5 g disodium phosphate, 2 g dextrose, 15 g agar.

2. Brain Heart Infusion Broth (Kasvi, Brazil): 37 g dissolved in 1 L of distilled water. Autoclave for 15 min at 121 °C. Composition (g/L): 10 g peptone, 7.5 g pork brain heart infusion, 5 g sodium chloride, 2.5 g dipotassium phosphate, 2 g dextrose.

2.3 Reagents for Vaccine Elaboration

1. Formaldehyde 36.9–37.1%. P.M.: 30.03 g/mol.

2. Hydrogen peroxide (H_2O_2) solution 30% in H_2O.

3. $1\times$ Phosphate buffer saline (PBS): Dissolve 8 g sodium chloride, 0.2 g potassium chloride, 1.44 g disodium hydrogen phosphate, and 0.24 g of disodium hydrogen phosphate in 1 L of distilled water (pH: 7.2–7.4). Autoclave for 15 min at 121 °C. Composition: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.

4. 1 M Hydrochloric acid (HCl).

5. 1 M Sodium hydroxide (NaOH). P.M.: 40.00 g/mol.

6. Freund Incomplete Adjuvant (FIA).

7. Aluminum hydroxide.

2.4 ELISA Reagents

1. 0.05% Tween-20 (v/v) (PBST): 0.5 mL of Tween-20 dissolved in 999.5 mL of PBS.

2. Carbonate bicarbonate buffer: One capsule dissolved in 100 mL of distilled water (pH: 9.6).

3. 1% bovine serum albumin (BSA) (w/v): 1 g dissolved in 100 mL of PBST.

4. Mouse anti-tilapia IgM monoclonal antibody IgG.

5. Peroxidase conjugated goat anti-mouse IgG.

6. Tetramethylbenzidine (TMB).

7. 2 M Sulfuric acid (H_2SO_4).

2.5 Equipment

1. Incubator shaker.

2. Centrifuge.

3. Spectrophotometer.

4. Microplate reader.

3 Methods

3.1 Vaccine Preparation

3.1.1 Formaldehyde-Inactivated Bacteria

1. Prepare a pre-culture of the *S. agalactiae* strain in BHI plates at 28 °C, for 48–72 h (*see* **Note 1**).

2. Transfer *S. agalactiae* colonies into 300 mL BHI broth and incubate at 28 °C, for 72 h at 150 rpm.

3. Prior to inactivation, calculate the bacterial number by plate count (*see* **Note 2**).

4. Dilute the bacterial suspension in 200 mL of sterile PBS (*see* **Note 3**).

5. Add formaldehyde to the suspension in order to obtain a final concentration of 3% and incubate at 4 °C, for 24 h.

6. Centrifuge the suspension at $10,000 \times g$, for 10 min (4 °C) and discard supernatant and resuspend the pellet in 120 mL of sterile PBS for inoculum preparation (repeat step "f" three times) (*see* **Note 4**).

7. Discard supernatant and adjust the final volume of the vaccine by adding PBS ($OD_{540nm} = 1.256$).

8. Check sterility by plating 100 µL of the inactivated inoculum onto BHI plates and incubate at 28 °C, for 72 h.

9. Store the vaccine at 4 °C until use.

3.1.2 H$_2$O$_2$-Inactivated Bacteria

1. Prepare a pre-culture of the *S. agalactiae* strain in BHI plates at 28 °C, for 48–72 h.

2. Transfer *S. agalactiae* colonies into 300 mL BHI broth and incubate at 28 °C, for 72 h at 150 rpm.

3. Prior to inactivation, calculate the bacterial number by plate count (*see* **Note 2**).

4. Add H_2O_2 to the suspension in order to obtain a final concentration of 5% and incubate at 4 °C, for 6 h (*see* **Note 5**).

5. Centrifuge the suspension at $10,000 \times g$, for 10 min (4 °C) and discard supernatant and resuspend the pellet in 120 mL of sterile PBS for inoculum preparation (repeat step "f" three times).

6. Discard supernatant and adjust the final volume of the vaccine by adding PBS (OD_{540nm} of 1.256).

7. Check sterility by plating 100 µL of the inactivated inoculum onto BHI plates and incubate at 28 °C, for 72 h.

8. Store the vaccine at 4 °C until use.

3.1.3 Inactivation by pH Manipulation

1. Prepare a pre-culture of the *S. agalactiae* strain in BHI plates at 28 °C, for 48–72 h.

2. Transfer *S. agalactiae* colonies into 300 mL BHI broth and incubate at 28 °C, for 72 h at 150 rpm.

3. Prior to inactivation, calculate the bacterial number by plate count (*see* **Note 2**).

4. Add 1 M NaOH to the suspension and adjust to a pH 10.0 and incubate at room temperature for 3 h (*see* **Note 6**).

5. Add 1 M HCl to the suspension and adjust to a pH 7.0 (*see* **Note 6**).

6. Dilute the bacterial suspension in 200 mL of sterile PBS (*see* **Note 3**).

7. Add formaldehyde to the suspension in order to obtain a final concentration of 3% and incubate at 4 °C, for 24 h.

8. Centrifuge the suspension at $10,000 \times g$, for 10 min (4 °C) and discard supernatant and resuspend the pellet in 120 mL of sterile PBS for inoculum preparation (repeat step "f" three times).

9. Discard the supernatant and adjust the final volume of the vaccine by adding PBS (OD_{540nm} of 1.256).

10. Check sterility by plating 100 µL of the inactivated inoculum onto BHI plates and incubate at 28 °C, for 72 h.

11. Store the vaccine at 4 °C until use.

3.2 Vaccination and Challenge

1. Anesthetize the fish by immersion in benzocaine solution (100 mg L^{-1}).

2. Administer the vaccine by intraperitoneal injection (0.1 mL).

3. Return the fish to their respective tanks with aerated water.

4. Keep the temperature at 28 °C using heaters and control water quality parameters with daily water changes (10%).

5. Evaluate the efficacy of the developed vaccine after 28 days post immunization by an intraperitoneal challenge with 0.1. mL of *S. agalactiae* (1.02×10^5 CFU mL^{-1}) and using the formula RPS = [1 − (% mortality in vaccinated fish/% mortality in control fish)] × 100 [21].

3.3 Inclusion de Adjuvants in a H$_2$O$_2$-Inactivated Vaccine

1. Emulsify the H_2O_2-inactivated vaccine with FIA at a ratio of 1:1 (*see* **Note 7**).

2. Mix the H_2O_2-inactivated vaccine with a 0.5% sterile aluminum hydroxide solution (w/v) at a ratio of 1:1.

3. Stored the adjuvanted vaccines at 4 °C until use.

3.4 Determination of Anti-S. agalactiae IgM Antibodies

1. Determine the antibody titer of the immunized Nile tilapias serum samples by ELISA in 96-well microtiter plates.

2. Coat the required number of wells in the microtiter plates with a suspension containing 10^7 CFU (100 µL per well) and left overnight at 4 °C (*see* **Note 8**).

3. Wash three times with 300 µL of PBST.

4. Block for 1 h at room temperature with 1% BSA and repeat Subheading 3.3, **step 3**.

5. Dilute each serum sample (1:25) using PBST as diluent, and add 100 µL of the mixture to each well (*see* **Note 9**).

6. Incubate at 25 °C for 3 h and repeat Subheading 3.3, **step 3**.

7. Dilute mouse anti-tilapia IgM monoclonal antibody Ig G (1:33) using PBST as diluent, and add 100 µL of the solution to each well.

8. Incubate for 1 h at 25 °C, and repeat Subheading 3.3, **step 3**.

9. Dilute peroxidase conjugated goat anti-mouse IgG (1:3000) using PBST as diluent, and add 100 μL of the solution to each well (*see* **Note 10**).

10. Incubate for 1 h at 25 °C, and repeat Subheading 3.3, **step 3**.

11. Add 100 μL of TMB substrate to each well.

12. Stop the ELISA reaction after 15 min with 50 μL of 2 M H_2SO_4 to each well.

13. Read the OD of the reactions at 450 nm in a microplate reader.

14. Measure the relative amount of specific antibody as the OD value.

4   Notes

1. Before the beginning of the experiment, the *S. agalactiae* stocks used for the vaccine elaboration were reactivated in BHI plates at 28 °C, for 48–72 h. After that, the isolates were reactivated *in vivo* by three passages in Nile tilapia. For that, a group of fish were injected intraperitoneally with 0.1 mL of the reactivated bacteria strain and monitored daily for clinical signs and mortality. Then, dead and moribund fish were removed and samples were aseptically obtained from brain, head kidney and spleen to isolate the bacteria. Finally, the steps were repeated twice.

2. Prior to inactivation, the bacterial number must be calculated by plate count. In the end, this valor must be adjusted taking into account the final volume of the inactivated vaccine. In our study the final concentrations were: 1.54×10^9 CFU mL^{-1} for both the formaldehyde-inactivated bacteria and H_2O_2-inactivated bacteria and 5.28×10^9 CFU mL^{-1} for the vaccine by pH manipulation.

3. In order to guarantee full inactivation previous to the addition of formaldehyde, we added 200 mL of sterile PBS to the 300 mL bacterial suspension for increasing the volume of formaldehyde that will be added. In the end, we have a 500 mL bacterial suspension where is added 15 mL of formaldehyde (3% bacterial suspension).

4. Before centrifuging, the 500 mL bacterial suspension was distributed into 50 mL conical centrifuge tubes (6 tubes). After centrifugation, 20 mL of PBS was added to each tube in order to resuspend the pellet (three times).

5. Before use, sterilize the stock solution of H_2O_2 by using a 0.22 μm membrane filter.

6. In order to avoid contamination during inactivation by pH manipulation, a duplicate flask with the same bacterial

concentration should be prepared. In this flask must be added the volume needed of each reagent (1 M NaOH and 1 M HCl) to obtain the desired pH. After knowing which are the exact quantities to modify the pH, add the reagents to the flask where the test will be developed.

7. Prepare the emulsion in a 50 mL Falcon tube. Add the adjuvant in the tube first. Then, while vortexing, add the inactivated bacterial suspension and continue vortexing vigorously for 15 min until a thick emulsion forms. The tubes must be filled at its maximum capacity to avoid air entrance.

8. The suspension containing 10^7 CFU is obtained as it follows:
   (a) Prepare 10 mL of *S. agalactiae* suspension dissolved in PBS (adjusted to turbidity of 0.5 McFarland, equivalent to a concentration of 10^8 UFC/mL.
   (b) Centrifuge for 10 min at 4 °C (10,000 × g) and discard supernatant.
   (c) Add to the pellet a volume of 10 mL (9.95 mL of PBS and 0.05 mL of 10% neutral buffered formalin).
   (d) Incubate for 24 h at 4 °C.
   (e) Centrifuge the suspension at 10,000 × g, for 10 min (4 °C) and discard supernatant and resuspend the pellet in 10 mL of PBS (repeat this step twice).
   (f) After that, discard the supernatant and add 10 mL of carbonate/bicarbonate buffer.
   (g) Dispense 100 μL of the solution in each well of the microtiter plate.
   (h) Incubate overnight at 4 °C.

9. The appropriate dilution of the serum (1:25) was obtained in previous assays. Firstly, we calculated the cut off of the positive control as the mean absorbance plus three standard deviation of the negative control by using several dilutions (1:25, 1:50, 1:100).

10. The appropriate dilution of peroxidase conjugated goat anti-mouse IgG should be determined by varying dilutions of the antibodies prior to obtain the optimal values.

Acknowledgments

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Part VII

Vaccines Against Ticks

Chapter 15

Anti-Tick Vaccines: Current Advances and Future Prospects

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Abstract

Ticks are increasingly a global public health and veterinary concern. They transmit numerous pathogens that are of veterinary and public health importance. Acaricides, livestock breeding for tick resistance, tick handpicking, pasture spelling, and anti-tick vaccines (ATVs) are in use for the control of ticks and tick-borne diseases (TTBDs); acaricides and ATVs being the most and least used TTBD control methods respectively. The overuse and misuse of acaricides has inadvertently selected for tick strains that are resistant to acaricides. Furthermore, vaccines are rare and not commercially available in sub-Saharan Africa (SSA). It doesn't help that many of the other methods are labor-intensive and found impractical especially for larger farm operations. The success of TTBD control is therefore dependent on integrating all the currently available methods. Vaccines have been shown to be cheap and effective. However, their large-scale deployment for TTBD control in SSA is hindered by commercial unavailability of efficacious anti-tick vaccines against sub-Saharan African tick strains. Thanks to advances in genomics, transcriptomics, and proteomics technologies, many promising anti-tick vaccine antigens (ATVA) have been identified. However, few of them have been investigated for their potential as ATV candidates. Reverse vaccinology (RV) can be leveraged to accelerate ATV discovery. It is cheap and shortens the lead time from ATVA discovery to vaccine production. This chapter provides a brief overview of recent advances in ATV development, ATVs, ATV effector mechanisms, and anti-tick RV. Additionally, it provides a detailed outline of vaccine antigen selection and analysis using computational methods.

Key words Anti-tick vaccines, Anti-tick vaccine antigens, Computational biology, Tick control, Reverse vaccinology

1 Introduction

Climate change continues to affect peoples' behaviors, environmental changes, and exerts differential effects on socioeconomic factors. Unfortunately, this appears to be altering tick population dynamics, biodiversity, prevalence, and driving the invasion of tick species into new areas globally [1–3]. Ticks are increasingly becoming a global veterinary and public health concern; they feed off

animals and transmit a number of pathogens of veterinary and public health importance [4–6]. This is particularly important for developing countries that have recently adopted the use of improved germplasm to increase the productivity of their indigenous livestock breeds and in the process reduced their inherent tick and tick-borne disease (TTBD) resistance [7]. Coincidentally, the public health provision systems in sub-Saharan Africa (SSA) are already overburdened and not amenable to the emerging and re-merging zoonotic diseases; ~20% of which are transmitted by various tick species [8]. Given the inelastic budgets of small holder livestock producers and non-resilience of SSA public health provision systems, TTBDs are of disproportionately higher impact to the veterinary and public health sectors in SSA.

Several TTBD control methods including acaricide use, breeding for resistance, tick hand picking, pasture spelling and anti-tick vaccines [ATVs] are in use; acaricide application and anti-tick vaccination being the most and least commonly used methods respectively [9, 10]. Increasing overuse and misuse of different acaricide molecules has led to selection of acaricide resistant tick strains which has limited the efficacy of acaricide tick control [11]. Moreover, the ever-increasing acaricide prices in tick endemic SSA implies that over reliance on acaricide TTBD control is not sustainable. Sustainable TTBD control is therefore premised on integration of the currently available TTBD control methods [12].

Anti-tick vaccination using recombinant *R. microplus* gut antigens; Bm86/Ba86 and Bm95 demonstrated significant (55–100%) reduction in *R. microplus, B. annulatus* and *B. decoloratus* strains from Latin America, Australia, Iran, Africa, Israel and the United States of America. Field trials with *Gavac™; a* Bm86-based vaccine commercially available in Latin America, controlled infestations (by reducing tick feeding and egg laying) with *R. microplus and B. annulatus* and reduced babesiosis transmission [13–15]. The Bm95 gene sequence is non-variable compared to the highly variable Bm86 gene sequence. Thus, a Bm95-based anti-tick formulation protects against Bm86 resistant tick strains, hence promising to be a better antigen for tick Boophilus/Rhipicephalus species from different parts of the world. Anti-tick vaccination results in significant (>60%) reduction in the frequency (by over 30 days) of acaricide application and the cost (reduction by ~20 USD/animal/year) of TTBD control [14]. As such, this approach is not only environmentally benign but is likely to be amenable to resource constrained smallholder livestock producers in SSA. These studies have underscored the potential of integrating anti-tick vaccines into mainstream tick control strategies and the possibility of cross-tick species protection by vaccines as a sought after added value.

Advances in genomics and proteomics have allowed a number of tick antigens to be identified and explored for the development of anti-tick vaccines [ATVs]. Additionally, reverse vaccinology [RV]

offers ways of shortening the process of anti-tick vaccine antigen (ATVA) discovery using computational methods [16, 17]. With these omics (vaccinomics, proteomics, genomics, etc.) advances, one would expect a large number of commercial ATVs to be made available. This is especially puzzling because vaccines are cheap, environmentally benign and have been shown to be efficacious in controlling ticks [18]. Possible drawbacks to development of ATVs include accessibility of antigens i.e., easily exposed antigens in the saliva and midgut as opposed to less accessible antigens such as structural proteins. This is exacerbated by limited understanding of ATV effector mechanisms and a very large cost of testing ATVs in large ruminant models [18, 19]. In this chapter, we provide a brief overview of recent advances in ATV development, most promising ATVAs, ATV effector mechanisms and anti-tick RV. This will help in understanding the different computational biology-based ATVA screening algorithms that we deem necessary in accelerating ATV development, translation and testing. For details of advances in ATV development and RV please refer to; [20–23].

1.1 Overview of Recent Advances in Anti-Tick Development

Traditional vaccinology methods have been used for close to a century in the development of protozoa, bacteria and virus vaccines but also in an attempt to develop anti-parasitic (including anti-tick) vaccines. In the case of ATVs, the initial steps involve screening of antigens delivered from crude homogenates of tick internal organs like salivary glands to control tick infestations and tick-borne diseases. Traditional vaccinology methods suffer from reasonably low precision and require large amounts of time and financial investment especially during initial antigen screening and validation before candidate antigens can be successfully constituted into vaccines [24–26]. The last half of the four decades of ATV development has been facilitated by advances in molecular biology techniques which have allowed for studies that explore hosts, vector and parasite interaction. Such studies have made it possible to discover new molecules that can be explored as ATVAs capable of disrupting tick-pathogen cycles. Central to these advances are new gene sequencing and annotation (bioinformatics) techniques, that underpin deployment of RV and systems biology approach (otherwise called vaccinomics) to hasten proteome/genome screening for ATAs and their subsequent evaluation as ATVAs [22, 26–28].

1.2 Current Promising Anti-Tick Antigens and Commercial Vaccines

ATA discovery is premised on the deep understanding of tick biology; so that once such antigens are inoculated into the host, they elicit tick biology-disruptive antibodies. A series of tick biological functions have been targeted in ATA discovery namely, tick attachment and on-host feeding, water balance, blood digestion, heme and iron metabolism, detoxification of xenobiotics, vitellogenesis, and fertility (Fig. 1). Studies that have led to the discovery of different ATAs have recently been summarized in more
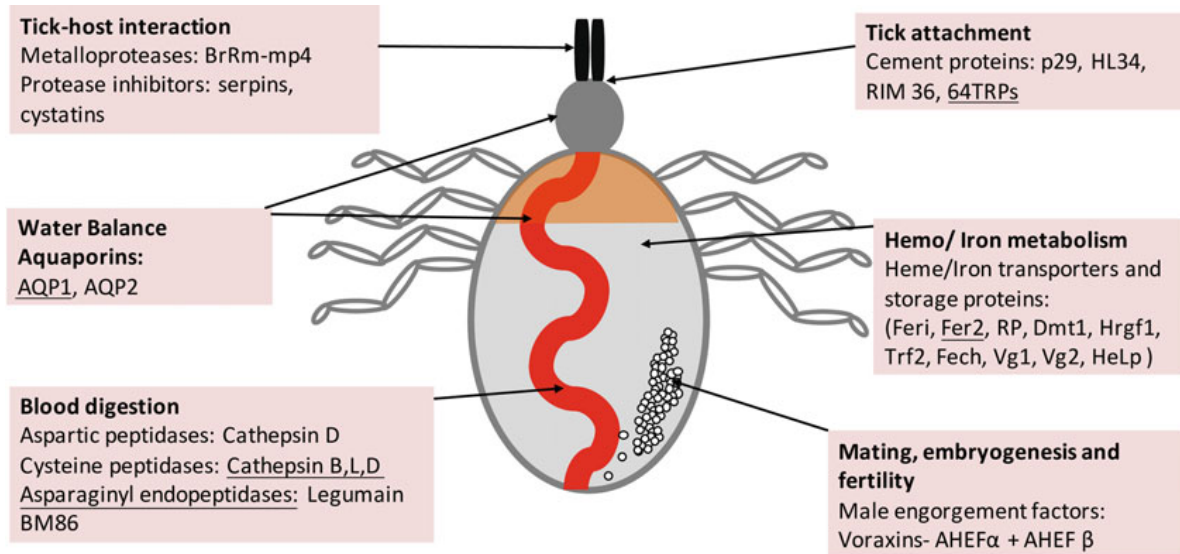
Fig. 1 Most promising anti-tick-antigens. The underlined antigens are the most promising ATVAs pending further evaluation

extensive reviews and technical meeting proceedings [22, 26, 28, 29]. However it suffices to present here an overview of the most promising ATAs and commercially available ATVs.

The collagen-like protein P29, HL 34 protein rich in tyrosine and proline residue repeats, tick cement derived RM 36 protein and a secreted 15 kDa protein called 64P are able (to a large extent) to disrupt either tick attachment or feeding. Of these, the truncated 64P constructs (64TRPs) with glutathione S-transferase (GST) is the most immunogenic. It causes *R. sanguineus* and *I. ricinus* midgut rapture and blocks transmission of tick-borne encephalitis in laboratory animal models. As well, the 64TRPs are promising broad spectrum ATA subject to successful testing in large ruminant animal models [30–32].

Tick saliva is a complex milieu of bioactive compounds that keep the host's innate immune responses at bay so that ticks feed and fully engorge. Despite the huge diversity of these bioactive compounds, only a few of them; metalloproteases and protease inhibitors have shown high potential as ATAs. Recombinant metalloprotease HLMP1 in combination with serine proteases (serpins) causes varying levels of tick mortality, reduction in tick engorgement and egg laying, and up to 60% efficacy [22]. They are however met with a problem of redundancy that can be overcome by only targeting their non-redundant epitopes as explained later in this chapter.

Blood meal concentration (osmoregulation) and digestion are essential to tick survival and reproduction. This makes tick aquaporins (water channels), acidic peptidases (cathepsins), and exopeptidases of the papain type; enzyme systems responsible for blood mean digestion, very promising targets for ATV development.

However, just like tick sialome proteins, the tick blood digestion enzyme system is associated with very high levels of redundancy and thus hard to exploit for ATV development using recombinant technology. This problem is likely to be overcome by advances in vaccinomics and RV discussed later in this chapter. It is only recombinant aquaporins 1 (e.g., from *I. ricinus*) that has proved 50–75% efficacious in disrupting *I. ricinus* and *R. microplus* female tick engorgement respectively [33, 34] . These results prompted our recent efforts that led to *in silico* identification of three highly conserved and antigenic tick aquaporin 1 peptide motifs [35]. Recombinant and aquaporin 1 peptide motifs are therefore promising AVAs that warrant further testing in different geographical setups and animal models respectively [22, 35].

Detoxification and sequestration of toxic substances including xenobiotics, heme, reactive oxygen and other toxic moieties are essential biological processes in ticks. Non-heme iron is isolated by ferritin 1 (Fer1) and transported from the tick gut to peripheral tissues by Fer2. Fer2 is a non-redundant highly immunogenic midgut protein; better ATA compared to Fer1 which shows a great level of redundancy. Fer2 and GST (heme and xenobiotics detoxification) formulations reached overall efficacy of 60–98% for the control of *Rhipicephalus spp.;* Fer2 being the most efficacious [36–38].

The last category of antigens that have been explored for incorporation into ATV formulations are derived from proteins that regulate tick mating (tick engorgement factor), egg yolk deposition and fertility (Vitellogenin enzymes). Although single recombinant proteins in this category did not sufficiently protect animals from tick infestation or reduce oviposition and egg hatching [39–42], a cocktail vaccine constituted of two recombinant vitellogenin enzymes (Boophilus yolk pro-cathepsin and cathepsin L-like vitellogenin degrading cysteine endopeptidase) and recombinant GST (metabolic detoxifying enzyme) showed improved efficacy [43]. This indicates that multi-antigen ATVs might provide enhanced effect against ticks and tick-borne hemoparasites (TBHs); an endeavor RV is likely to accelerate [22].

It is desirable that ATVs be effective against multiple tick and tick-borne hemoparasite (TTBH) species. To formulate multi-tick and TBH-effective vaccines, the antigens in such formulations need not only be very highly antigenic and immunogenic but also non-redundant (coded for by single genes), target TTBH essential biological functions (for survival or transmission), expressed in multiple tick species and should be accessible to specific antibodies [27, 44]. To act against multiple ticks and TBH species, ATVs need to be constituted of multiple anti-tick antigens (ATAs). It is for these reasons that not many ATVs have been formulated since the initial proof of concept more than four decades ago [25]. As such, there are only two commercial ATV formulations registered for Latin America (Gavac) and Australian markets (TickGARD) [18].

Despite their huge technical efficiency (>50% acaricide usage reduction, babesiosis, and anaplasmosis incidence reduction and being environmentally benign] in the control of *R. microplus*, Gavac and TickGARD have not been marketed beyond the Latin American and Australian markets. Inefficacy of these vaccines against some tick species and competition with acaricide markets are some of the technical reasons why these vaccines have not succeeded as anticipated. For details of other problems with commercialization of Gavac and TickGARD *see* Ref. 18. It has been suggested that a systems biology approach would ensure selection of antigens that would offer protection against different TTBHs hence solving some of the inefficacy-related problems. As well, this would further reduce the frequency of acaricide application hence improving ATV uptake [22]. Later in this chapter, we suggest and discuss TTBH antigen selection algorithms that can be used to formulate multi-TTBH vaccines.

1.3 Overview of Anti-Tick RV

RV involves a rational process of identifying requisite tick specimens, generating their transcriptomes using available sequencing methods, identifying candidate ATAs from these transcriptomes and evaluating their antigenicity and allergenicity using a battery of bioinformatics-based methods [22, 27] (see ATA screening algorithms later in this chapter). These candidate ATAs are further evaluated using wet bench-based methods. The wet bench evaluations often involve the manipulation of target genes and observation of subsequent phenotypic consequences; resulting from interference with the biological functions of such target genes. The commonly used genetic interference (knockdown) methods include random or insertional mutagenesis, RNA interference, homologous recombination among others [22, 23]. Thereafter, the best individual or combinatorial candidate ATAs are evaluated for their anti-tick effects in tick capillary feeding, laboratory animals, and cattle vaccination field trials before they are formulated into ATVs (Fig. 2).

However, there is a general lack of fully annotated tick genomes making it hard to rapidly screen for ATAs using RV. Tick genomes have not been widely sequenced owing to their enormous size (in some instances >7 Gb), high number of repetitive sequences and genes with unknown functions [22]. To explore ATA discovery, notwithstanding the current drawbacks associated with the general paucity of tick genomes, the publicly available *I. scapularis* genome/proteome (ABJB010000000/UniProt ID: UP000001555) can initially be screened for candidate ATAs. These candidate ATAs can then be related back to the genes and where available, these genes can be screened individually for tick species of interest and where unavailable these genes from ticks isolated from geographical locations of interest can be sequenced.
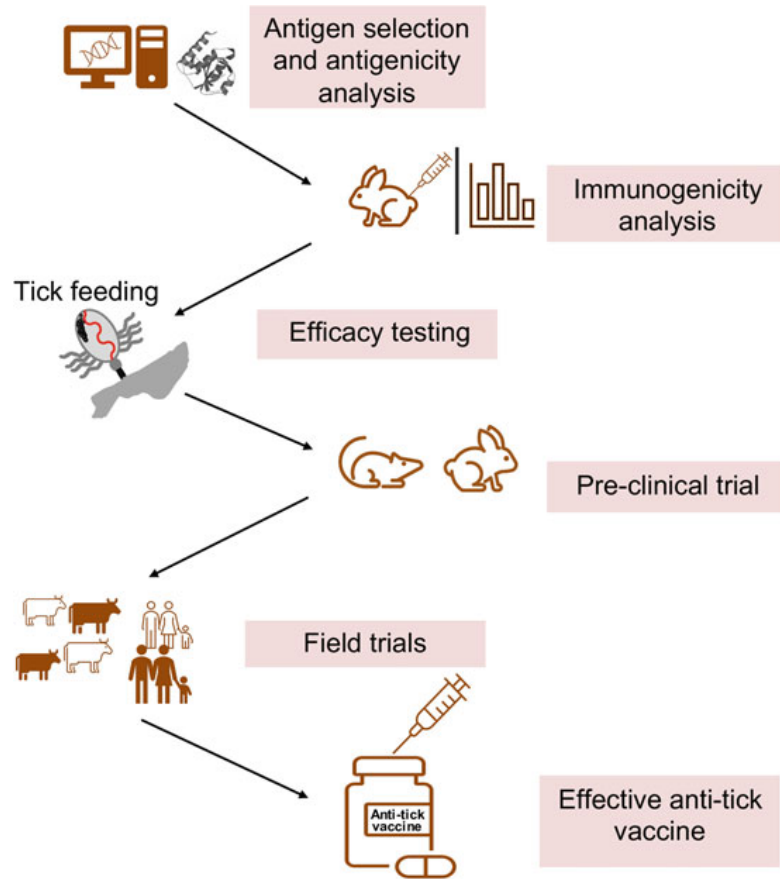
Fig. 2 Key anti-tick vaccine reverse vaccinology steps

The future of ATVs will benefit from different tick species' genome sequencing and annotation given the advances in gene sequencing technologies and the renewed interest in ATV development stimulated by increasing tick acaricide resistance. As more tick genomes become available, molecular biology and omics advances will contribute to refining conformational B-cell epitopes and narrowing down the range of candidate epitopes before downward evaluations. In addition, these advances will lead to improved understanding of tick-gene function by predictions of subcellular localizations of gene products and resultant phenotypes upon knockdown of such genes [22, 23]. Together, these advances and the ability to tandemly render these techniques to both TTBH genomes; a systems biology approach (otherwise called vaccinomics) can be taken to accelerate development of multi-TTBH-effective vaccines [22, 27, 28].

1.4 Overview of Anti-Tick Effector Mechanisms: Gaps and Future Prospects

The effector (cellular, molecular and immune) mechanisms by which the only commercially available BM86/BM95 anti-tick vaccine or the promising antigens in different ATV pipelines are not well characterized. However, the protection offered by BM86/BM95-based vaccine has been reported as the elicitation of

BM86/BM95 (of other promising antigens for that matter) antigen-specific antibodies in ticks that interfere with tick feeding (the biological function of BM86/BM95 gene products [22, 24]. This explanation does not offer, details of how the antibody–antigen interactions occur and how this interrupts feeding or how such interactions can be enhanced to augment the well documented acquired resistance (involving antibodies, complement, antigen presenting cells, lymphocytes and other bioactive molecules) to ticks by livestock [19, 45]. If these molecular or cellular interactions are well characterized, current advances in RV would provide means by which these interactions could be enhanced to further improve vaccine performance. Reverse vaccinology-guided hypotheses that help anticipate and explain effector mechanisms of potential ATVs can be used to accelerate ATV development [46].

2   Computational Methods in Anti-Tick Vaccine Development

2.1   General Considerations

There have been significant advances in tick genomics, proteomics and transcriptomics. There are now some tick genomes and proteomes that are publicly available [47, 48]. The increasing abundance of freely available information offers several research opportunities particularly for rational driven computational methods such as RV. This is a key development that is important in overcoming some of the bottlenecks faced in conventional ATV design. There is a near complete proteome for *I. scapularis* available from UniProt (Proteome ID: UP000001555) that comprises 20,473 proteins. Additionally, several studies have reported proteins in the tick sialome (salivary gut transcriptome) and mialome (midgut transcriptome) [49, 50]. It is important to have a sound rationale and validation for selecting a suitable protein target as outlined in Subheadings 1.2 and 1.3. An ideal candidate ATA should be; vital, important for the tick biological function, sufficiently different from the host protein repertoire to allow minimal cross-reactivity, should be immunogenic enough to produce immune products such as antibodies or activate host T-cells.

2.2   Retrieving Protein Sequence Data

Protein sequences for the target proteins can be downloaded from UniProt (https://www.uniprot.org/) or Refseq (https://www.ncbi.nlm.nih.gov/refseq/) in *fasta* format. UniProt (release 2020_02) contains 22,172 *Ixodidae* proteins out of which 109 have been curated and evaluated by Swiss-prot. Refseq (Release 201) contains 33,352 *Ixodidae* proteins.

1. Where a structure for the protein is available it can be downloaded from Protein data bank (PDB) in PDB format (https://www.rcsb.org/). PDB contains 66 *Ixodidae* protein structures (accessed on August 12, 2020). It is important to determine if

the obtained crystal structure has missing residues or not. Depending on the localization of the missing residues, subsequent analysis maybe difficult or even impossible. In such cases, homology modeling may be used to model the missing regions.

2. Given the small number of available *Ixodidae* protein crystal structures in PDB (66 out of 20,473) it might be necessary to calculate an accurate template-based homology model where possible. Online modeling engines such as HHPRED only require the amino acid sequence in *fasta* format as the input (https://toolkit.tuebingen.mpg.de/tools/hhpred).

2.3 Amino Acid Sequence Analysis

Initial analysis of target amino acid sequences includes identifying important factors that determine immunogenicity of a protein, such as protein cellular and subcellular localization, sequence similarity of the candidate tick protein with those from the host protein repertoire, physicochemical properties such as hydrophobicity/hydrophilicity, and molecular weight.

1. Determine protein localization and function by uploading the fasta sequence of the query protein in the online subcellular localization predictors such as, CELLO (subCELlular LOcalization predictor) (http://cello.life.nctu.edu.tw/) [51] or CELLO2GO: A Web Server for Protein subCELlular LOcalization Prediction with Functional Gene Ontology Annotation (http://cello.life.nctu.edu.tw/cello2go/) [52].

2. Perform a Multiple Sequence Alignment (MSA) in order to determine the level of similarity between the tick vaccine protein candidate and any mammalian host proteins.

3. Perform a BLASTp (*see* **Note 1**) (Basic Local Alignment Search Tool) search using the tick protein candidate as a query against potential host taxids (*see* **Note 1**). BLAST provides users with the option of selecting specific organisms prior to performing a search. This option should be used to ensure that a BLAST search conducted will only yield mammalian host proteins. Provide the input protein sequence in fasta format using the standard IUPAC amino acid residues or using the NCBI Databank accession number.

4. The sequences that show high sequence identities and coverage should be downloaded in a fasta format.

5. This *fasta* file downloaded in (b) will then be used to carry out a MSA using programs such as MUSCLE (Multiple Sequence Comparison by Log- Expectation) [53]. MSA with MUSCLE can be performed either on a local computer or on a European molecular biological laboratory webserver (https://www.ebi.ac.uk/Tools/msa/muscle/). It is advisable to use different

multiple sequence alignment programs and compare the results. This allows for the identification of the most accurate and reliable alignment.

6. Visualization of the MSA can be done using Jalview software. In Jalview the MSA can be colored by Clustalx to show conserved amino acids. Quantitative and qualitative measurements (Sequence identity and Sequence similarity) may be used to assess the degree of conservation in the dataset used. For example, the MSA results can be better visualized using a protein dissimilarity matrix using R packages such as *AlignStat* [54]

7. A structural comparison of the query protein structure or homology model with any homologous host proteins can be carried out by superimposing the two proteins in order to calculate the Root Mean Square Deviation (RMSD) score. In PyMOL, a score close to zero indicates that the protein structures are highly similar. To perform the structural alignment using PyMOL, use the "align" functionality after loading the two proteins (https://pymolwiki.org/index.php/Align).

2.4 Identification of Peptide Motifs that Are Unique to Tick Protein Sequences

In cases where there is some sequence similarity between the tick and host proteins, motif discovery methods can be used to identify short tick peptide sequences (motifs) that are unique to ticks. This allows for differential targeting of the tick protein for peptide-based vaccine design. There are a number of motif discovery algorithms but in this chapter, we shall describe motif identification with Multiple Em for Motif Elicitation (MEME) suite Version 5.1.1 web-server [55] (http://meme-suite.org/tools/meme).

1. Upload the MSA *fasta* file generated in Subheading 2.3, **step 2** for Motif identification with Multiple Em for Motif Elicitation (MEME suite V 5.1.1) webserver (http://meme-suite.org/tools/meme) (*see* **Note 2**). The diversity and number of sequences used in the dataset will highly affect the quality of the discovered motifs. As a result, a fairly large and diverse sequence dataset is recommended for this analysis.

2. MEME suite provides users with the option of selecting the number as well as the length of the motifs to be detected. It is important to consider the minimum length for potential peptide-based vaccine candidates. These two parameters should be adjusted until an optimal result is obtained. Insignificant motifs can be identified using the Pearson correlation provided with MEME suite output.

3. MEME suite outputs are mainly 3 types of files (graphic HTM, and two text files [MEME and MAST text file]).

2.5   Motif Mapping on the Protein 3D Structure

Motif mapping is important in demonstrating the localization of the motifs on the protein structure. This has implications on peptide vaccine development. For example, for transmembrane proteins, motifs located in the transmembrane domains might not be ideal targets as compared to those located on extracellular domains.

1. Mapping of the predicted motifs onto the protein 3D structure can be done using PyMOL by rendering the target protein as either "cartoon" or as "surface" and then coloring the different peptide motifs.

2.6   Antigenicity Prediction of Tick Peptide Motifs

Antigenicity check is another important step in predicting the potential proteins'/peptides' ability to induce mammalian host immune responses. The main predictions of importance are host memory-associated immune responses such as T-cell or B-cell immune response [for T-cell prediction, this chapter will focus on MHC class II prediction (*see* **Note 3**)].

2.6.1   T-Cell Receptor Binding Affinity Prediction

Studies have shown that hydrophobicity can be a good indicator of T-cell receptor binding of which MHC class I epitopes will be recognized by T-cells [56]. In this chapter we shall use ProPred [57] to demonstrate this.

1. To use ProPred, the amino acid query sequence is provided in FASTA, EMBL, or PIR file format (http://crdd.osdd.net/raghava/propred/). The user has to specify the type of the input file format provided.

2. Select single or multiple alleles from the available 51 HLA-DR alleles. Choose the output format, set the threshold value and submit. Due to the possibility of obtaining false positives, a threshold value above 3% is not advisable.

3. Four possible output formats can be selected, html I, html II, graphical and tabular. All four methods will show peptide length and score for each peptide (MHC I epitope) that binds to a given HLA allele.

2.6.2   B-Cell Receptor Binding Affinity Prediction

B-cell epitopes are those that are recognized by B-lymphocytes and immunoglobulins (antibodies). They can be classified as continuous or discontinuous (*see* **Note 4**). Continuous (linear) epitopes are predicted based on the hydrophilicity, amino acid protein charge, secondary structure, b-turn propensity, secondary structure prediction of the input sequence amino acid, frequency of the amino acid in the experimentally determined B-cell epitopes, and surface accessibility. Discontinuous (non-linear) epitope prediction requires the protein 3D structure to predict based on the query protein surface topology. In this chapter, we shall give approaches for both linear and discontinuous epitope prediction using Discotope [58] and BepiPred [59] respectively.

1. Discotope   (http://www.cbs.dtu.dk/services/DiscoTope/instructions.php) is based on the query proteins 3D dimension to determine surface accessibility and propensity scale matrix.

   (a) Upload the input file, which is usually the amino acid coordinate (pdb file format) or the PDB accession number of the protein in question and submit.

2. BepiPred carries out a sequence-based analysis using Hidden Markov Models (HMM) and propensity scale methods.

   (a) Upload a *fasta* sequence of the query protein to the webserver (http://tools.iedb.org/bcell/) and submit.

      The pipeline outputs a graphical html file and maps the antigenic regions on the entire protein sequence and scores for each amino acid.

3   Notes

1. In tick vaccine development, a protein BLAST (BLASTp) of any potential tick target protein should include the mammalian hosts of interest e.g., *Bos taurus*: taxid:9913 or *Homo sapiens*: taxid:9605. Additionally, other tick species can be included to gauge the conservation of the protein in other ticks.

2. During motif discovery, for anti-tick vaccine development, the input sequence should include any highly similar sequences from other tick species and any similar mammalian host protein sequences. The ultimate goal is to get peptides that are unique to ticks and where possible allow a means of targeting more than a single tick species.

3. Unfortunately, there is still limited success with the MHCII predictors due to insufficient model training data, difficulties in identifying 9-mer peptides (which constitute the majority of the MHC binding peptides), and relative permissiveness of the binding groove of MHCII molecules unlike MHC class I predictors. MHC class I binding peptides activate cytotoxic T-cells, which generally respond to the intracellular pathogens (e.g., viruses).

4. Discontinuous B-cell epitopes have been demonstrated to constitute the vast majority of natural B-cell binding peptides unlike linear B-cell peptide. B-cell peptide predictions are therefore advised to focus on discontinuous peptides where possible.

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Vaccines Against Vector-Borne Diseases

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Abstract

Arthropod vectors account for a number of animal and human diseases, posing substantial threats to health and safety on a global scale. Ticks are considered as one of the most prominent vectors, as they can parasitize almost any vertebrate class and transmit a multitude of infectious diseases, particularly ones that affect humans and domestic animals. While various tick species elicit different tick-borne infections in specific geographic regions, single species can have widespread effects, such as blacklegged ticks, which are widely distributed across the eastern United States and can transmit a variety of infections, including Lyme borreliosis, anaplasmosis, relapsing fever disease, ehrlichiosis, babesiosis, and Powassan virus disease. Despite increasing awareness about ticks as serious disease vectors, effective vaccines against most tick-borne infections are not available. Previously, the successful development of an anti-tick vaccine for use in veterinary animals was based on an 86-kDa midgut antigen from *Rhipicephalus* (formerly *Boophilus*) *microplus* ticks. Herein we describe the fundamentals of vaccine development using protein antigens as model vaccinogen candidates, beginning with the cloning, expression, and purification of recombinant proteins, host immunization, and the assessment of protective efficacy in laboratory settings using a tick-borne murine model of Lyme borreliosis.

Key words Vector-borne diseases, Anti-tick vaccines, Bm86, Murine models, Gut antigens

1 Introduction

Vector-borne diseases, which represent a leading cause of many zoonoses, impart substantial mortality and morbidity in domestic animals and humans [1]. Tick-transmitted infections are particularly on the rise, yet they remain difficult to control due to a lack of effective preventive measures, such as vaccines. Depending on the geographical region, tick-borne infections often constitute the primary source of vector-borne diseases in humans and animals. For example, approximately 95% of the reported cases of vector-borne infections in the United States were associated with ticks, which transmit at least 15 serious diseases in the country, including Lyme disease [2]. In fact, Lyme disease is now known to occur in more than 80 countries and is considered as the most prevalent vector-

borne disease in the Northern Hemisphere. Many arthropod-borne infections are associated with a lack of proper diagnosis and effective antimicrobials. Combatting the diseases via vector control remains difficult due to the absence of successful insecticides or acaricides, as well as the emergence of drug resistance in relevant vectors or parasites, as encountered with many mosquito-borne infections. Current anti-tick measures, which include tick avoidance, protective clothing, and tick repellents, are only 20–40% effective [3]. All of these factors underscore the unmet need for the development of effective preventive measures, such as vaccines, against arthropod-borne infections, which is the focus of many research laboratories.

Host protection against tick-borne pathogens and other infections can depend on memory responses and/or neutralizing antibodies, which are primarily directed against one or more antigens from the concerned pathogen, most notably against membrane antigens. The development of effective vaccines largely relies on the selection of key target antigen(s) that are essential to pathogen persistence, microbial virulence, and pathogenesis, which remains as one of the greatest challenges in vaccine studies. Care must be taken when selecting a target vaccine candidate; as observed in several infectious agents, the misdirection of the immune response toward immunodominant but non-protective surface epitopes can compromise the efficacy of a vaccine. Therefore, after experimental testing, scientists typically choose an antigen (while incorporating an effective combination of adjuvant) that directs the most effective host immune responses via the genesis of protective memory responses, and/or antigens that elicit antibodies against the most neutralizing epitopes. Important determinants for the design of a vaccinogen would ideally include the selection of an essential antigen from a given pathogen that is conserved among target microbial species, is highly immunogenic, and features expression on the cell surface [4–6]. In addition to microbial antigens that can induce protective immunity in immunized hosts, selected proteins from the vector that are essential to either the arthropod life cycle or blood meal engorgement, or that support pathogen persistence in the arthropod, can also serve as targets for novel anti-vector vaccines, including anti-tick vaccines [7–10]. For example, a gut protein from the cattle tick *Rhipicephalus* (formerly *Boophilus*) *microplus*, called Bm86, which is expressed in the tick gut during feeding, has been commercially developed as an anti-tick vaccine [11–13]. The immunization of cattle with recombinant Bm86 has been shown to induce effective protection in cattle against *R. microplus* infestation and the transmission of associated tick-borne infections. Herein, we detail the general procedures for the production and assessment of vaccine candidates and the assessment of their efficacy in animal models.

2 Materials

2.1 Selection of Vaccine Candidates

1. Standard computer and access to literature and publicly available databases, such as PubMed.

2.2 Cloning and Expression of Recombinant Proteins from E. coli

1. Polymerase chain reaction (PCR) components like nuclease free water, reaction buffer, Deoxynucleotide Triphosphates (dNTPs), primers (10 μM), DNA template, DNA polymerase, Dimethyl sulfoxide (DMSO), pipettes, filter pipette tips, PCR tubes or PCR plates, ice bucket, microcentrifuges, and thermocycler.

2. Agarose, Tris-base, Acetic acid and EDTA (TAE) buffer, weighing scale, Erlenmeyer flask, microwave oven, and Ethidium Bromide (EtBr).

3. Agarose gel electrophoresis chamber, DNA molecular weight marker, loading dye, casting tray, comb, buffer tank, tank lid, power cables, and power supply.

4. Personal protective equipment (PPE), plastic wrap, and ultra-violet (UV) transilluminator.

5. Commercially available PCR purification kit.

6. Restriction enzyme(s) and buffer, ligase buffer, T4 DNA ligase, Bovine Serum Albumin (BSA), microcentrifuges, and biological incubator.

7. Commercially available DNA gel extraction kits, sterile razor blade, weighing scale.

8. Competent cells, SOC or Luria broth (LB), shaking biological incubator, LB agar plates, antibiotics, and plastic spreader.

9. Antibiotics, inducers, sterile culture tubes, conical centrifuge tubes, and spectrophotometer.

10. SDS-PAGE running buffer, supplies for SDS-PAGE setup (including glass plates, casting frame, casting stand, comb, gel apparatus, and power supply), SDS sample buffer, protein molecular weight marker, Milli-Q water, Coomassie Blue R250, methanol, and acetic acid.

2.3 Purification of Recombinant Proteins

1. Commercially available protein purification reagents, such as glutathione-S-transferase (GST) or poly-histidine (His) affinity purification reagents.

2. Common salts like sodium phosphate and sodium chloride, imidazole, lysozyme, phosphate-buffered saline (PBS), sonicator, and various centrifuge machines and tubes.

2.4 Immunization of Mice

These studies require the approval and guidance of the appropriate Institutional Biosafety Office and the Institutional Animal Care and Use Committee of the researcher's organization.

1. Antigen of choice.
2. Adjuvant (such as alum or Freund's adjuvant).
3. PBS.
4. Two glass tuberculin syringes, A and B.
5. Plastic connector joint.
6. Syringe and needle for injections.

2.5 Vaccination and Challenge Studies

1. Biosafety Level 2 materials for working with blood-borne pathogens, ticks, and mice (as recommended by the Institutional Biosafety Office and Institutional Animal Care and Use Committee).
2. Culture of *Borrelia burgdorferi*, biological incubator, BSK media, and Petroff-Hausser counter slide.
3. Centrifuge.
4. Materials for the maintenance of ticks: environmental chamber, *Ixodes scapularis* nymphs, white sticky mat, and paintbrush.

3   Methods

3.1 Selection of Vaccine Candidate

As discussed in the Introduction, the vaccine candidate should be selected after performing extensive empirical analyses and literature surveys to identify an ideal immunogenic antigen that induces protective host immunity. The antigen should be produced in a recombinant form using a suitable expression system. For example, if the target native antigen is a posttranslationally modified protein (such as a glycoprotein), an appropriate eukaryotic expression system should be used, such as arthropod or mammalian cell lines. For bacterial proteins, we routinely use *Escherichia coli*-based expression systems, which are described below (*see* **Note 1**).

3.2 Cloning and Expression of Recombinant Proteins from E. coli

1. Select a desired vector for protein expression and specify the cloning site. Make sure the preferred restriction enzymes do not recognize the DNA sequence of the target insert. The restriction enzymes can be chosen to be active in the same buffer. One can use available restriction enzyme analysis software tools.
2. Carefully design the primers for the amplification of the target DNA sequence. Ideally, primers should contain, at both ends, a sequence of 3 to 6 nucleotides to facilitate restriction enzyme binding, the restriction enzyme site in 5′ to 3′ orientation, and a 10–20 nucleotide sequence complementary (forward primer)

Table 1
A typical reagent setup for a PCR reaction

Components	50 μL reaction	Final concentration
Sterile, nuclease free water	Add up to 50 μL	
Reaction buffer (10×)	5 μL	1×
dNTPs (10 mM)	1 μL	200 μM each
Forward primer (10 μM)	x μL	Up to 1 μM
Reverse primer (10 μM)	x μL	Up to 1 μM
Template DNA	x μL	Variable (typically up to 250 ng)
DNA Polymerase	x μL	Typically 0.5 to 2 unit
DMSO—optional	Up to 1.5 μL	Up to 3%
Final Volume	50 μL	

or reverse complementary (reverse primer) to the amplified region. There are many online software programs that can be used for primer design.

3. Set up a PCR (Table 1). The reaction conditions vary by specific template, primer, and other conditions. An example protocol is as follows: thaw all the reagents (if necessary), briefly centrifuge for a few seconds, and keep them on ice. For a typical 50 μL reaction, add the following components in PCR tubes (*see* **Note 2**):

4. Mix well, spin down, and transfer the samples to the thermocycler.

5. Set up the PCR amplification (*see* **Notes 3** and **4**). Routine thermocycling conditions for two- and three-step PCR are indicated (Table 2).

6. Detect the PCR products in an agarose gel. Prepare the running buffer using the following protocol: Make a stock solution (50×) of TAE by dissolving 242 g of Tris-base in 500 mL of double distilled water (ddH$_2$O). Add 57.1 mL of 100% acetic acid and 100 mL of 0.5 M sodium EDTA (pH 8.0). Adjust the volume to 1 L using ddH$_2$O. Prepare 1 L of 1× TAE solution by diluting 20 mL of stock 50× TAE into 980 mL of ddH$_2$O.

7. Prepare the agarose gel (1% concentration). First, weigh 1 g of agarose. Add the agarose to an Erlenmeyer flask containing 100 mL of 1× TAE. Swirl the flask and place it in a microwave to melt the agarose. Microwave for 45 s, remove the flask, and swirl it again to mix the components well. Repeat this process until the agarose is completely dissolved. Let the agarose mixture cool down to about 50°C. Add EtBr to a final concentration ~ 0.5 μg/mL to stain the gel. Mix well (*see* **Note 5**).

Table 2
An example of optimal thermocycling conditions

Cycle step	Two-step PCR		Three-step PCR		Cycles
	Temp.	Time	Temp.	Time	
Initial Denaturation	95–98 °C	30 s to 5 min	95–98 °C	30 s to 5 min	1
Denaturation	95–98 °C	10 s to 1 min	95–98 °C	10 s to 1 min	25–35×
Annealing	–	–	x °C	10–45 s	
Extension	68–72 °C	30 s to 1 min/kb	68–72 °C	30 s to 1 min/kb	
Final extension	72 °C	5–10 min	72 °C	5–10 min	1
Hold	4 °C	∞	4 °C	∞	

8. Cast and run the gel using an electrophoresis apparatus according to the manufacturer's instructions. Slowly load 5–10 μL of DNA molecular weight marker of the appropriate size into the first lane of the gel. Mix a small fraction of the DNA samples with the concentrated loading dye and load them slowly into the following lanes. Cover, assemble, and run the gel electrophoresis using ~80 V–150 V, until the loading dye reaches at least the lower half of the gel (or up to the desired length) (*see* **Note 6**).

9. Analyze the gel using an ultraviolet (UV) transilluminator, wearing a UV protective shield. Typically, a single band of the amplified DNA template should be clearly visible on the agarose gel. If satisfied, purify the amplified DNA from the PCR reaction and continue with the digestion. One can use commercially available PCR purification kits (*see* **Notes 7** and **8**).

10. Digest your purified PCR product (insert) and the plasmid vector (*see* **Note 9**). A typical 50 μL digestion reaction is indicated (Table 3).

    Add these listed components into a sterile microcentrifuge tube. The restriction enzyme(s) should be added last. Mix well and centrifuge briefly. Incubate the reaction based on the recommended conditions, temperature, and time. After incubation, proceed with agarose gel electrophoresis analysis. Identify the desired DNA bands and excise them with a sterile razor blade. Extract and purify the DNA from the agarose gel, using available DNA gel extraction kits. Proceed with ligation as in the step below, or store the DNA samples at −20 °C for later use (*see* **Notes 10–13**).

11. Prepare for the ligation reaction of the plasmid vector and PCR insert. For a successful ligation reaction, the vector and the insert should be present in an optimal molar ratio. A molar ratio of 1 vector: 3 insert and an amount of 100 ng vector are

Table 3
A typical reagent setup for restriction digestion reaction

Components	Single digestion	Double digestion
Sterile, nuclease free water	38 μL	37 μL
Restriction Enzyme 10× Buffer	5 μL (1×)	5 μL
DNA, typically 1 μg/μL	1 μL	1 μL
BSA 10× (optional)	5 μL (1×)	5 μL
Restriction Enzyme—A, 10 u/μL	1 μL	1 μL
Restriction Enzyme—B, 10 u/μL	–	1 μL
Final Volume	50 μL	50 μL

Table 4
A typical reagent setup for DNA ligation reaction

Components	Ligation reaction
Sterile, nuclease free water	Add up to 10 μL
Vector DNA	~100 ng
Insert DNA	x ng
Ligase buffer 10×	1 μL (1×)
T4 DNA Ligase enzyme	x μL (per manufacturer)
Final Volume	10 μL

typically used. Online calculators can be used to estimate the molar ratio and quantify the correct amount of the digested DNA products needed for the ligation reaction.

12. Thaw the digested insert, digested vector, and ligase reaction buffer. Centrifuge all the reagents for several seconds in a microcentrifuge and keep them on ice.

    A typical 10 μL ligation reaction is indicated (Table 4).

13. Incubate at room temperature for 2 h or at 16 °C overnight, or following instructions in the product user manual. Consult the manufacturer's recommendations to optimize the incubation conditions, time, and temperature.

14. If necessary, verify the ligation by agarose gel electrophoresis analysis, or directly proceed with the transformation step below, or store the ligated products at −20 °C for later use (*see* **Notes 14–16**).

15. Prepare for the transformation of DNA to competent *E. coli*. Thaw the competent cells and keep them on ice for 20–30 min. Thaw the ligated DNA and the control samples, centrifuge for

several seconds in a microcentrifuge, and keep them on ice (*see* **Note 17**).

16. Transfer the LB agar plate(s) containing the proper antibiotic (s) from 4 to 37 °C.

17. Mix 2–3 μL of the DNA with 50–100 μL of competent cells per transformation and keep them on ice for 30 min.

18. Heat shock the mixture for 90 s in a 42 °C waterbath and place it back on ice for 2 min.

19. Add 900 μL of SOCor LB media without antibiotic and allow the transformed cells to grow on a 37 °C shaking incubator for 1 h.

20. Remove the cell culture and the LB agar plate(s) from 37 °C and spread 100–200 μL of the transformed cells on the LB agar plate(s) using a plastic spreader.

21. Keep the plate(s) at room temperature for ~5 min to dry and incubate them at 37 °C overnight. The following morning, check the plate(s) for the presence of colonies (*see* **Notes 18** and **19**).

22. Perform an *E. coli* colony screening to confirm the presence of the desired insert in the transformed cells (*see* **Note 20**).

23. Using a sterile pipette tip, pick up 4–6 distinct colonies, transfer them into PCR tubes, proceed with colony PCR, and sequence the amplified DNA to confirm its identity (*see* **Notes 21** and **22**).

3.3 Expression and Purification of Recombinant Proteins from E. coli

First, optimize the induction conditions for the desired protein using the following strategy:

1. Remove the transformed cells from the −80 °C freezer and keep them on dry ice. Pick up a small amount of the culture using a sterile pipette tip (and return the frozen culture to −80 °C). Spread the cells on an LB agar plate with antibiotic (s), grow overnight and the next day pick up a single colony and mix it with ~5 mL of LB media, containing the proper antibiotic(s).

2. Shake the culture overnight at 37 °C. The following day, transfer a part of the growth culture (at 1:50–1:100 dilution) to ~50 mL of secondary LB culture containing the appropriate antibiotic(s), and grow in a 37 °C shaking incubator.

3. Assess the culture growth with OD_{600} measurements. Allow the cells to grow, typically for ~1–6 h, until they reach mid-log growth phase (an OD of 0.6–0.8).

4. Add the proper inducer at different concentrations. Incubate at various temperatures (16–37 °C) for time periods ranging from 1 h to overnight. Identify the optimal induction

conditions based on the presence of the induced protein band on an SDS-PAGE gel (*see* **Notes 23** and **24**).

5. Prepare the gel according to the following protocol (or use a premade or commercially available gel). Mix 30 g of Tris-base, 144 g of glycine, and 10 g of SDS in 1 L of H_2O, in order to prepare a 10 x SDS-PAGE running buffer of pH 8.3. Dilute the buffer to 1x concentration for electrophoresis. Next, assemble the gel cassette in the casting frame and place it in the casting stand. Use a pipette to load water in the space between the glass plates to verify that there is no leakage. Then, prepare the resolving gel, vortex gently, pour it into the gel cassette, and wait ~30 min for it to get polymerized. Prepare the stacking gel, vortex gently, and pour it into the gel cassette. Wait ~30 min for the gel to polymerize before use (*see* **Notes 25–27**). Below is the recipe for a typical 12% resolving gel and 4% stacking gel (Table 5).

6. Run the SDS-PAGE gel. Carefully remove the comb from the stacking gel and place the casting frame in the electrophoresis chamber. Fill the casting frame and chambers with $1\times$ running buffer. Prepare the samples by mixing ~10–20 μL of sample aliquot with the SDS sample buffer to reach a final concentration of $1\times$ SDS buffer. Heat the samples at 95 °C for 10 min. Slowly load ~2 μL of the molecular weight marker at the first lane, followed by ~10 μL of each sample at the next lanes. Run the gel using a voltage of 80–180 V, depending on the setup. After running the gel, stain the gel using 0.2–0.5% Coomassie Blue R250 in 50% methanol and 10% acetic acid and de-stain in 50% methanol and 10% acetic acid. Staining and de-staining can be done for a minimum of 30 min. Proceed with the purification step (*see* **Notes 28** and **29**).

Table 5
A typical reagent setup for SDS-PAGE gel

Components	Stacking gel 4%	Resolving gel 12%
Deionized water	9 mL	5.03 mL
30% Acrylamide/bis-acrylamide	1.98 mL	6.0 mL
Tris–HCl (0.5 M, pH 6.8)	3.78 mL	–
Tris–HCl (1.5 M, pH 8.8)	–	3.75 mL
10% SDS	150 μL	150 μL
TEMED (N,N,N',N'-tetramethylethylenediamine)	15 μL	7.5 μL
10% Ammonium persulfate (APS)	75 μL	75 μL
Final Volume	15 mL	15 mL

3.4 Purification of Recombinant Proteins (See Note 30)

1. The purification of recombinant proteins depends on the fusion tag used for protein expression in *E. coli*. For example, a protein with a GST tag or His tag would bind to the respective ligands, such as glutathione coupled to sepharose beads, or nickel-charged resins for affinity-based protein purification. These reagents are available from a variety of commercial vendors and can be used according to specific manufacturer protocols. The following is a general description of typical protein purification steps. Before proceeding with purification, it is important to determine if the protein is soluble or associated with an insoluble fraction of *E. coli* cells (*see* **Note 31**).

2. Centrifuge a 500 mL culture (or other desired volume) at 8000 × *g* for 10 min at 4 °C.

3. Remove the supernatant and add ice-cold PBS. Centrifuge at 8000 × *g* for 10 min at 4 °C and remove the PBS.

4. Use a sterile pipette or scraper to harvest the cells into a 50 mL tube. Lyse a small amount of those cells to locate the protein and store the rest at −80 °C.

5. For lysis, add 5 mL of 20 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole, pH 7.4, and 1 mg/mL lysozyme. Keep on ice for 30 min.

6. Sonicate the mixture on the ice bath for ~5 min. Set the sonicator to ~50–70% capacity and the pulses to 10 s on and 10 s off. Properly submerge the sonicator tip into the cells and avoid the formation of excessive foam.

7. Centrifuge the lysate at 3000 × *g* for 15 min and separate the supernatant from the pellet.

8. Take a small amount from each fraction, run an SDS-PAGE gel to locate the protein, and determine the purification conditions accordingly. If the protein is in the insoluble fraction, use detergent-based lysis conditions, which are available from specific commercial purification reagents and protocols. If the protein is in the soluble fraction, move forward with native purification steps.

9. Add the lysate to a resin (such as nickel-chelating resin) and bind using gentle agitation for 30–60 min.

10. Settle the resin by gravity or centrifugation (at 800 × *g* for 2 min) and carefully aspirate the supernatant. Store the supernatant at 4 °C for SDS-PAGE. Wash the resin with the appropriate buffer. Repeat this step three times or as needed.

11. Elute several times with the elution buffer and analyze with SDS-PAGE (*see* **Note 32**).

12. If possible, verify the protein's identity via western blot analysis, using antibodies against the protein or against its binding affinity tag (*see* **Note 33**).

13. Aliquot and store the purified protein at $-80\,°C$ (*see* **Note 34**).

3.5 Immunization of Mice (see Note 35)

1. Prepare the immunization or booster shot. Screw one glass tuberculin syringe with a plunger (syringe A) into a plastic connector joint. Screw a second glass tuberculin syringe without a plunger (syringe B) into the second opening of the connector joint. Turn the white knob of the connector joint down toward the blue spiraled knob.

2. Add the appropriate amount of adjuvant to syringe B. Make sure the syringe is pointing up; this is easily achieved by leaning the syringe apparatus against a hard surface. Add PBS, or the appropriate amount of protein mixed with PBS, into syringe B. Slowly pull the plunger from syringe A until the entire sample is in syringe A. Remove syringe B. Place the plunger into syringe B. Gently remove all air from syringe A, then place syringe B back into the plastic connector joint.

3. Emulsify (i.e., mix) the sample in the syringes, as described above, for 10–15 min, then store the syringes at $-20\,°C$ for 5 min. Take the syringes out of the freezer and mix for 5–10 min. Check the sample for proper emulsification by testing it on water; the sample will not dissolve in water if prepared correctly. This process may take 30–60 min.

4. Remove one of the syringes and the plastic connector. Gently place a needle on the remaining syringe.

5. For a typical mouse injection, one would need at least 10 μg of protein to immunize a single animal, prepared in 50–100 μL of adjuvant. The steps below can be followed as an example schedule (*see* **Note 36**).

6. Day 1: Immunize mice with either the protein or PBS mixed/adsorbed with an adjuvant (such as alum or Freund's adjuvant), in a 1:1 volume mixture.

7. Day 15 (14 days after first immunization): Boost mice with either the protein or PBS mixed with adjuvant.

8. Days 21–31: Collect blood samples between Days 21–25, and analyze the serum for the presence of antibody formation via western blot. On Day 31 (14–15 days after the second immunization), boost mice with either the protein or PBS mixed with adjuvant.

9. Days 40–45: Collect blood samples and analyze the serum for the presence of antibody formation via western blot. Titer the antibodies in the serum using ELISA, following any standard protocol.

3.6 Vaccination and Challenge Studies

Once the animals are immunized with a vaccine candidate protein and the development of high-titer antibodies has been confirmed, challenge studies can be designed to assess the vaccine potential of the immunogen. If the vaccine candidate is a tick antigen, one can challenge with a group of ticks to evaluate any potential impacts of the immunized host-derived antibodies on tick infestation. If the immunogen is a microbial protein or a tick protein that blocks pathogen survival or transmission, the challenge studies could involve pathogen-infected ticks. The following examples describe key steps in vaccination studies involving *B. burgdorferi* and *Ixodes* ticks (*see* **Note 37**).

*3.6.1 Assessment of the Vaccine Candidate Protein for the Prevention of Pathogen Acquisition in the Vector (see **Note 38**)*

1. Immunize animals, such as mice, with recombinant vaccine proteins, as detailed in the above paragraphs.

2. Infect the mice with a particular pathogen, such as *B. burgdorferi* via subcutaneous needle inoculation. Count *B. burgdorferi* cells using a Petroff-Hausser counter slide and inject 10^3–10^5 cells/mouse.

3. Two to three weeks after inoculation, collect sera from the immunized animals.

4. Perform immunoblots with *B. burgdorferi* lysates and mouse sera to confirm infection.

5. Challenge the infected and immunized animals with naïve *Ixodes* nymphs (5–15 ticks/mouse, 5 mice/group).

6. Collect ticks after 48–72 h of feeding, and after full repletion.

7. Evaluate the pathogen levels within the ticks via qPCR, using pathogen-specific primers. Any deficiency in pathogen levels after engorgement on immunized mice will confirm the potential vaccine effects of the immunized protein.

3.6.2 Assessment of the Vaccine Candidate Protein for the Prevention of Pathogen Transmission to Hosts

1. Immunize animals, such as mice, with recombinant vaccine proteins, as detailed in the above paragraphs.

2. Challenge naïve immunized animals with *B. burgdorferi*-infected *Ixodes* nymphs (3–5 ticks/mouse, 5 mice/group).

3. Collect fed ticks at various time points of blood meal engorgement, after 12–72 h of feeding, and after full repletion.

4. Evaluate the pathogen levels within the ticks via qPCR, using pathogen-specific primers.

5. Euthanize mice two to four weeks after tick feeding, and collect tissue biopsy samples (from the skin, joint, and urinary bladder) to measure spirochete burdens by qPCR, serology (immunoblotting using *B. burgdorferi* lysates), and tissue culture in BSK medium. Any deficiency in the pathogen levels of immunized mice (in terms of negative qPCR, serology, and culture) will confirm the potential vaccine effects of the immunized protein.

*3.6.3 Assessment
of the Vaccine Candidate
Protein for the Prevention
of Tick Infestation*

1. Immunize animals, such as mice, with recombinant vaccine proteins, as detailed in the above paragraphs.

2. Challenge naïve immunized animals with naïve *Ixodes* nymphs (10 ticks/mouse, 3–7 mice/group, as determined by a statistical power analysis).

3. Monitor the progress of tick feeding by counting the ticks that have dropped from the mice on a daily basis, until all have detached.

4. Count and weigh the detached ticks using a laboratory scale.

5. Allow ticks to molt and calculate their molting rates two months after repletion. Any deficiencies in the number of attached or engorged ticks, the feeding weights of ticks that engorged on immunized mice, or their molting rates will confirm the potential anti-tick vaccine effects of the immunized protein.

4   Notes

1. All laboratory work in research institutions should be performed with the appropriate training by responsible authorities, as well as the approval, supervision, and guidelines of the relevant Department of Environmental Safety, Sustainability & Risk.

2. Some PCR reactions require extensive optimization to achieve the desired amplification of the specific target, such as when DNA contains higher levels of secondary structures. In some reactions, the final concentration of the primer can vary from 0.2 to 1.0 µM. We normally use proofreading (high-fidelity) DNA polymerases for cloning purposes. The user should refer to the manufacturer's recommendations for the final concentrations of reagents, including DNA polymerase, since the enzyme activity/units may vary. The DMSO can be used for PCR with GC-rich templates. If there are multiple samples, calculate the final volume needed for each reagent and prepare a master mix. Do not add the DNA template or primers when the templates or target sequence are different.

3. Determine the annealing temperature according to the melting temperature (T_m) of the primers. Online T_m calculators can be used. A temperature gradient can be used to further optimize the annealing step.

4. The two-step PCR protocol, which combines the annealing and extension steps, can be used when the primer T_m values are close to the extension temperature (≤ 72 °C) or in case of certain real-time PCR applications.

5. Prepare the agarose gel in the proper concentration to improve the separation of the DNA molecules and the excision of the desired gel band. The concentration is expressed as weight per volume (w/v) and usually varies within 0.5–2%. Higher concentrations are preferred for small DNA molecules, while lower concentrations are used for larger DNA molecules. The agarose gel can be alternately stained in running buffer containing 0.5 μg/mL of EtBr for 20 min after the end of electrophoresis, followed by the de-staining step in running buffer for 20 min. EtBr is a potent mutagenic factor and needs to be handled and disposed of appropriately. Protect your eyes and your skin, and always wear PPE. Alternatively, SYBR Safe DNA Gel Stains, which are less hazardous than EtBr, can be used.

6. The loading dye is usually made at 6× concentration. It should be mixed with the DNA samples and/or with the running buffer at 1× final concentration.

7. If there is nonspecific amplified DNA or poor amplification, reassess the template quality and optimize the PCR parameters.

8. Proceed with the digestion step or keep the DNA samples at −20 °C.

9. Thaw the insert, the plasmid vector, and the restriction enzyme buffers. Centrifuge all reagents for several seconds and keep on ice. Remove the restriction enzyme(s) from the freezer and add to the reaction. If the restriction enzymes are active in the same buffer, prepare each digestion reaction in the same tube for both enzymes. If the enzymes are active in different buffers, sequential digestions can be set up for each of the enzymes. For sequential digestions, purify the digested DNA with a DNA purification kit before the second digestion reaction.

10. If necessary, prepare control reactions, which should include a reaction with a DNA template with a known cutting pattern, to verify the efficacy of the enzyme(s).

11. BSA can be added upon recommendation from the manufacturer for the enzyme's optimal activity. Higher amounts of DNA may require increased digestion time. Note that suboptimal reaction conditions can alter restriction enzyme specificity, a condition known as star activity.

12. For poor or incomplete digestion, ensure that the correct buffer is added, the reaction is incubated at the proper temperature for the appropriate time, a purified DNA template is used, and any type of contamination is avoided. In addition, verify that the cleavage is not blocked due to the use of methylation-sensitive enzymes. To avoid impaired digestion due to DNA methylation, DNA can be cloned in plasmids grown in dam−/dcm− competent *E. coli* cells.

13. To prevent self-ligation events, one can dephosphorylate the plasmid vector using DNA alkaline phosphatases, like the Calf Intestinal Alkaline Phosphatase (CIP). If phosphorylation is needed, the T4 Polynucleotide Kinase enzyme can be used.

14. If there is no ligation, optimize the molar ratio or the incubation conditions. Scale down the reaction volume when DNA concentrations are low.

15. A reaction with the cut vector alone plusligase can be used in transformation experiments, to validate adequate digestion of the vector and the prevention of vector re-circularization in case of phosphatase treatment.

16. A variety of cloning technologies, such as ligation-independent cloning (LIC), TA cloning, TOPO cloning, and Gateway cloning, have been developed to simplify and reduce the steps of routine cloning experiments. Those methods serve many different purposes, have numerous applications, and can be chosen according to the needs of each experiment.

17. A variety of competent cells with particular characteristics, such as DH5α *E. coli* cells, and different expression systems, including bacterial, yeast, insect, and mammalian cells, can be used according to a given experimental goal.

18. Increase the amount of cells that are spread on the LB plate(s), such as when a low number of colonies is expected. Centrifuge the culture at $5000 \times g$ for ~5 min (if using a microcentrifuge tube), remove most of the supernatant, thoroughly re-suspend the remaining medium (~200 μL), and spread on the plate(s).

19. Label the LB plate(s) on the bottom and incubate them upside-down to avoid contamination and direct water contact with the colonies.

20. As an alternative to the colony PCR screening method, transfer the colonies in Falcon tubes containing LB media with the proper antibiotic(s), and culture overnight on a 37 °C shaking incubator. Isolate the plasmid from the cells using commercially available plasmid extraction kits and proceed with the digestion, using the selected restriction enzymes while preparing the insert and the vector for ligation. Verify the size of the insert and the cut vector in an agarose gel. Extract the DNA from the band that is indicative of the insert and sequence it for verification.

21. Prepare control reactions to assess transformation efficiency. No colonies should be observed in plated cells containing the cut vector alone. If there are colonies, reassess the restriction/digestion steps or the stability of the antibiotic(s) in the plates. Colonies should be observed in plated cells containing the uncut vector, which would confirm cell viability and successful

transformation. If there are no colonies in the experimental plate, re-evaluate the restriction/digestion and ligation protocols and verify the efficiency of competent cells.

22. Prepare a glycerol stock(s) of the successfully transformed bacterial cells and store at -80 °C for long-term use.

23. As controls, prepare non-induced control culture(s).

24. Isopropyl β-D-1-thiogalactopyranoside (IPTG), which is a commonly used inducer for lac promoters, can be added in concentrations varying from 0.05 mM to 10 mM. L-arabinose can be used in a range of 0.001–0.4% for the induction of araBAD promoters.

25. Determine the appropriate concentration of the gel (4–20%) according to the molecular weight of the running samples. Typically, gels of lower concentration are preferred for samples of high molecular weight.

26. TEMED and APS should be the last reagents added during gel preparation, since the gel will begin to polymerize quickly.

27. Determine the appropriate volume of the resolving gel poured into the gel cassette, such that enough space remains for the stacking gel, usually a few centimeters below the comb. Fill this area with isopropanol (or water) to prevent the gel from drying during polymerization. Before adding the stacking gel, remove the isopropanol (or water) by angling the gel cassette.

28. Different formulations of the Coomassie Blue stains and other staining methods are available, including silver staining, fluorescent staining, and zinc staining, and can be used based on the experimental needs and downstream applications.

29. Confirm the presence of the induced protein by comparing with the control (non-induced) samples and determine the culture conditions that correspond to optimal yields based on the band size. If there is no induction, further modify the induced conditions, reassess the expression system or the cloning vector, and verify that there are no mistakes in the design and cloning of the DNA insert.

30. Insoluble proteins, present in the pellet fraction, require denaturation conditions for purification, e.g., the use of urea or guanidine hydrochloride. Different purification methods, such as gel filtration chromatography, ion-exchange chromatography, or affinity chromatography, can be used based on protein characteristics, including protein size, charge, and the presence of a specific affinity tag (His or GST).

31. During protein purification, store small portions from the defined steps. At the end, run a final SDS-PAGE gel to assess the efficiency of the purification method.

32. Colorimetric assays such as the Bradford Assay, the Lowry assay, and the BCA (Bicinchoninic Acid) protein assay, as well as NanoDrop spectrophotometers (measuring absorbance at 280 nm), can be used to quantify the protein.

33. In addition to western blot, other common protein detection methods, including ELISA, and even more sophisticated methods, such as mass spectrometry, can be used.

34. A good purification procedure should correspond to a high quantity and purity of the protein. A single band in an SDS-PAGE gel is indicative of a well-purified protein with no contaminants. If the protein is not well-purified or its final concentration is low, one can optimize the purification method or follow a different one.

35. All experimental protocols involving live vertebrate animals must be approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the researcher's organization.

36. Immunize mice subcutaneously, or via any other acceptable immunization routes.

37. Such studies have used *B. burgdorferi* strains and rodent models of Lyme disease, such as C3H mice, appropriately following IACUC and BSL-2 approval, compliance, and guidelines.

38. These types of vaccines might be relevant to the development of reservoir-targeted vaccines, in order to decrease pathogen persistence in wild hosts that maintain a given pathogen.

Acknowledgments

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Chapter 17

A Quantum Vaccinomics Approach Based on Protein–Protein Interactions

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Abstract

Vaccines are the most effective preventive intervention to reduce the impact of infectious diseases worldwide. In particular, tick-borne diseases represent a growing burden for human and animal health worldwide and vaccines are the most effective and environmentally sound approach for the control of vector infestations and pathogen transmission. However, the development of effective vaccines for the control of tick-borne diseases with combined vector-derived and pathogen-derived antigens is one of the limitations for the development of effective vaccine formulations. Quantum biology arise from findings suggesting that living cells operate under non-trivial features of quantum mechanics, which has been proposed to be involved in DNA mutation biological process. Then, the electronic structure of the molecular interactions behind peptide immunogenicity led to quantum immunology and based on the definition of the photon as a quantum of light, the immune protective epitopes were proposed as the immunological quantum. Recently, a quantum vaccinomics approach was proposed based on the characterization of the immunological quantum to further advance the design of more effective and safe vaccines. In this chapter, we describe methods of the quantum vaccinomics approach based on proteins with key functions in cell interactome and regulome of vector–host–pathogen interactions for the identification by yeast two-hybrid screen and the characterization by *in vitro* protein–protein interactions and musical scores of protein interacting domains, and the characterization of conserved protective epitopes in protein interacting domains. These results can then be used for the design and production of chimeric protective antigens.

Key words Vaccine, Interactomics, Music, Tick, Protective epitope, Immunology, Subolesin, Vaccinomics, Quantum vaccinology, Immunological quantum

1 Introduction

1.1 Vaccines and Vector-Borne Diseases

Vaccines are the most effective preventive intervention to reduce disease, disability, and death from a variety of infectious diseases [1]. According to the World Health Organization (WHO), vaccines currently prevent between 2 and 3 million deaths and protect many millions more from illness yearly (https://www.who.int/news-room/facts-in-pictures/detail/immunization). In particular,

vector-borne diseases (VBD) represent a growing burden for human and animal health worldwide and vaccines are the most effective and environmentally sound approach for the control of vector infestations and transmitted pathogens [2–6]. However, among the various limitations for the development of effective vaccines for the control of VBD is the combination of vector-derived and pathogen-derived antigens in vaccine formulations [6, 7].

Ticks (Acari: Ixodida) are obligate hematophagous arthropod ectoparasites that are second to mosquitoes as the most important vectors of pathogens causing diseases in humans and the first cause of VBD in farm animals [8]. Ticks constitute a model for the development of vaccines for the control of VBD [5–7]. Since the first and only vaccines against arthropod ectoparasites were registered and commercialized for the control of cattle tick infestations [9], leading research on tick vaccines has discovered new protective antigens using different methodological approaches in various tick species [10–12].

1.2 From Quantum Biology to Quantum Immunology

Biological systems are dynamical with constant exchange of energy and matter with the environment in order to maintain the state of non-equilibrium characteristic of living systems. Several mechanisms within living cells operate under non-trivial features of quantum mechanics such as quantum tunneling, which has been proposed to be involved in DNA mutation biological process [13–16]. Quantum tunneling is when particles with wave-like properties at the quantum scale can tunnel through apparently impermeable energy barriers with certain probabilities. After the discovery of the DNA double helix structure by Watson and Crick [17], Löwdin proposed the proton tunneling as the mechanism for point mutations in the DNA model [18]. These findings provided support for the discipline of quantum biology, which was originally proposed by Pascual Jordan in a book published in 1932 [19]. According to Marais et al. [14], "quantum biology should be defined in terms of the physical 'correctness' of the models used and the consistency in the explanatory capabilities of classical versus quantum mechanical models of a particular biological process." Indeed, recent evidences showed that living organisms may depend on the dynamics of small number of molecules such as proteins that are well localized (at nanometer scale) and operating over short time periods (in picoseconds), which support that nontrivial quantum mechanical processes play an important role in living systems before decoherence induced by surrounding environment can wash them out [13]. However, the area of quantum biology related to mutation requires experimental evidence that are difficult to obtain due to difficulties in their tractability to precise physical measurement contrasting incoherent random mutagenesis with quantum coherent mutagenesis [13]. Nevertheless, it is accepted that

quantum dynamics within living systems has been subjected to optimizing evolution, and life has learned to manipulate these quantum systems to its advantage in ways that need to be approached by future quantum biology studies [13, 20].

The development of the immune system contains random processes that are most obvious at the genetic level [21]. For example, the immune repertoire of lymphocyte response receptors is generated by somatic recombination and is different between individuals even if identical twins. However, the first finding supporting quantum immunology was reported by Germenis et al. [22]. The authors reported that atomic coordination is directly correlated to peptide immunogenicity as a direct relationship between peptide atomic/electronic structure and T cell receptors (TCR)-peptide bound to molecules of major histocompatibility complex (pMHC) functional avidity. If this ability involves some minimal determinism intrinsic to quantum mechanics, then it addresses the generic issue of protein–protein interactions. Recently, certain descriptors associated to the atomic structure of the peptide and its underlying electronic structure were associated with the immunological outcome of the *in vitro* TCR–pMHC interactions [23–25]. These results suggested that the study of the electronic structure of the TCR–pMHC interaction may leads to quantum immunology [22].

1.3 Quantum Vaccinomics

The characterization of protein–protein interactions and vaccinomics have been proposed as approaches for the development of vaccines for the control of tick infestations and tick-borne diseases (TBD) [10, 26]. In similarity with Albert Einstein's definition of the photon as a quantum of light [27], the immune protective epitopes were proposed as the immunological quantum [28]. Recently, a quantum vaccinomics approach was proposed based on the characterization of the immunological quantum to further advance the design of more effective and safe vaccines to target some of the challenges posed for ectoparasite control vaccines [29]. Our proposed pipeline for quantum vaccinomics consists of (a) the characterization of cell interactome and regulome in vector–host–pathogen interactions for the identification of proteins involved in the regulation of multiple biological processes through physical interactions with other proteins, (b) the identification by yeast two-hybrid screen and the characterization by multiple methodologies such as *in vitro* protein–protein interactions and musical scores of protein interacting domains, and (c) the characterization of conserved protective epitopes in protein interacting domains (Fig. 1). These results can then be used for the design and production of chimeric protective antigens [29] (Fig. 1).

The methodology for quantum vaccinomics proposed here focuses on proteins involved in cell interactome and regulome that function through protein–protein interactions for the
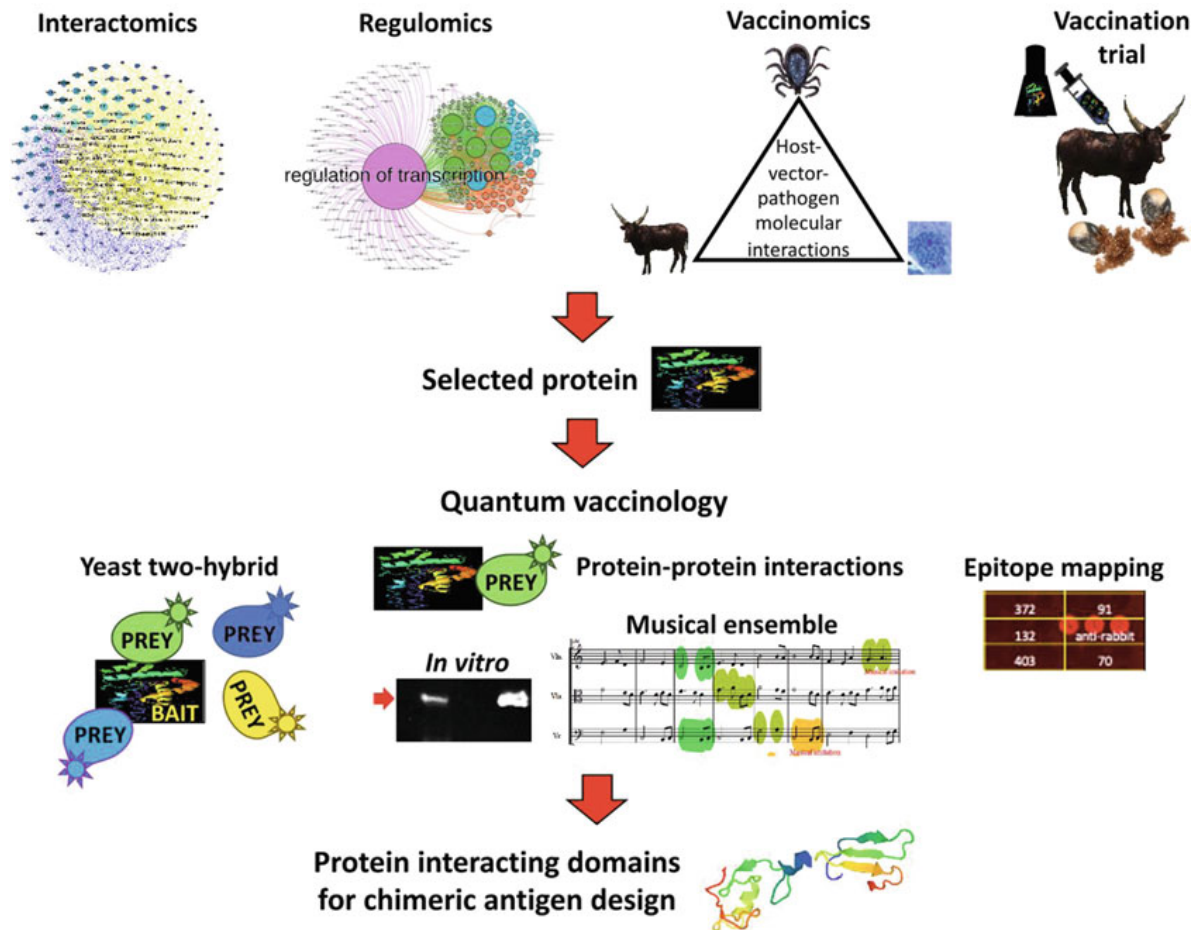
Fig. 1 Proposed pipeline for quantum vaccinomics. The characterization of the role of cell interactome and regulome in vector–host–pathogen interactions is used in a vaccinomics approach for the identification of proteins involved in the regulation of multiple biological processes through physical interactions with other proteins and with protective antigen capacity in vaccination trials. The selected protein is then used in quantum vaccinomics for the identification and characterization of protein interacting domains by Y2H and protein–protein interactions. Finally, the identification of protective epitopes in protein interacting domains is used for the design and production of chimeric protective antigens

regulation of multiple biological processes involved in vector–host–pathogen interactions. The rationale for selecting these proteins is that vaccination with protective epitopes in protein interacting domains will induce an antibody response not only interfering with protein translocation to the nucleus [30], but also blocking protein–protein interactions involved in the regulation of multiple biological processes [29]. As previously shown as a proof-of-concept for vaccines based on chimeric antigens containing Akirin/Subolesin protective epitopes (Q38 and Q41), vaccination with these antigens has proven efficacy for the control of different ectoparasites and infection by vector-borne pathogens [31]. Based on these results, herein we will use as a model protein, tick Subolesin (SUB) already identified as a protective antigen and involved in

protein–protein interactions to describe methods for the identification and characterization of protein–protein interactions, protein interacting domains and the characterization of protective epitopes.

2   Materials

All reagents used for buffer preparations need to be of analytical grade. The solutions are prepared with ultrapure water and stored at 4 °C, except for the solutions containing sodium dodecyl sulfate (SDS) that are stored at 20 °C to avoid detergent precipitation.

2.1   Reagents, Consumables, Kits, Equipment, and Software

These materials and their origin and use are described in Subheading 3.

2.2   Buffers

1. Transfer buffer. 25 mM Tris, 192 mM glycine, 0.02% SDS, 20% methanol. Prepare 10% SDS stock solution. Add 3.02 g of Trizma Base, 14.41 g of glycine and 2 ml of 10% SDS stock solution to 500 ml of water and mix. Add 200 ml of methanol and bring up the volume to 1000 ml with water. This buffer should be prepared fresh and refrigerate at 4 °C prior to protein transference.

2. Tris-buffered saline (TBS). 100 mM Tris, 0.15 M NaCl. Weigh 12.11 g of Trizma Base and 9 g of NaCl, add 500 ml of water and mix. Adjust pH to 7.5 with HCl. Bring up the volume to 1000 ml with water.

3. 3% bovine serum albumin (BSA) blocking solution in TBS. Add 1.5 g of BSA to 50 ml of TBS and mix with vortex until complete solubilization. This buffer should be prepared fresh prior to blocking the membrane.

4. Tris-buffered saline (TBS)-Tween buffer. 100 mM Tris, 0.15 M NaCl, 0.05% Tween-20. Prepare 10% Tween-20 stock solution. Add 12.11 g of Trizma Base and 9 g of NaCl to 500 ml of water and mix. Add 5 ml of 10% Tween-20 stock solution and adjust pH to 7.5 with HCl. Bring up the volume to 1000 ml with water.

5. 10 mM phosphate buffered saline (PBS). Weigh 0.26 g KH_2PO_4, 2.17 g $Na_2HPO_4 \cdot 7H_2O$, 8.71 g NaCl and add water to a volume of 1000 ml adjust to pH 7.4 with NaOH.

6. Standard buffer. Prepare 1 l PBS, pH 7.4 and add 0.05% Tween 20.

7. Blocking buffer. Prepare standard buffer with 1% BSA. Weigh 1 g BSA and add PBS to a volume of 100 ml.

8. Staining buffer. Prepare standard buffer with 10% blocking buffer. Add 50 ml blocking buffer to a volume of 450 ml of standard buffer.

9. Dipping buffer. 1 mM Tris, pH 7.4. Weigh 0.024 g and add water to a volume of 200 ml, adjust pH 7.4 with HCl.

2.3 Cultured Tick Cells

The *Ixodes scapularis* embryo-derived cell line ISE6 (provided by U. G. Munderloh, University of Minnesota, USA) is maintained in L-15B300 medium (300 ml L-15B medium plus 100 ml cell culture grade sterile water). The cells are cultured in sealed containers in ambient air at 31 °C, medium is changed once a week.

2.4 Antibodies from Vaccinated Cattle

Calves are vaccinated with vaccine formulations containing the SUB antigen. The animals are injected intramuscularly in the neck muscles with 2 ml vaccine (100 μg SUB per dose) on days 0, 30 and 60. Blood samples are collected after vaccination and serum immunoglobulins G (IgGs) purified using the NAb™ Protein G Spin Kit (Thermo Fisher Scientific) following the manufacturer's recommendation [32].

3 Methods

3.1 General Considerations

Different methodological approaches could be applied to the identification and characterization of protein interacting domains (*see* **Note 1**). However, these methodologies have been optimized for tick protein samples and are thus described here [33, 34].

3.2 Yeast Two-Hybrid (Y2H)

3.2.1 cDNA Library Construction

1. The *I. scapularis* embryo-derived cell line ISE6 is used for RNA extraction and cDNA library construction.

2. Extract total RNA from tick cells using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA, USA) and store them at −20 °C until used.

3. Evaluate total RNA quality using the Agilent 2100 Bioanalyzer RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA).

4. Perform cDNA synthesis and amplification using the Super SMART System™ principle (Clontech Laboratories, Mountain View, CA, USA) with adapted anchor primers containing unique SfiI restriction sites for directional cloning (SMART IV: 5′- AAGCAGTGGTATCAACGCAGAGTGGCCATG GAGGCCGGG -3′ and CDS III: 5′- ATTCTAGAGGCCTC CATGGCCGACATG(T)30VN-3′).

5. Polish the amplified cDNA using the protocol described by the SMART PCR cDNA synthesis manual (Clontech Laboratories).

6. Purify the cDNA using the DNA Extract II Kit (Macherey-Nagel, Düren, Germany).

7. Clone the cDNA in pACT2 plasmid via the SfiI sites.

8. Perform repetitive electroporation of *Escherichia coli* JM109 cells to obtain a library with a titter exceeding 3×10^7 cfu/ml.

3.2.2 Yeast Two-Hybrid Screen

1. Perform the yeast two-hybrid screen as described in the MATCHMAKER Two-Hybrid user manual (Clontech Laboratories).

2. Insert the full-length selected protein cDNA as a bait in the yeast expression vector pAS2-1 at NdeI and PstI sites.

3. Co-transform the yeast strain AH109 with plasmid pAS2-1-bait and pACT2 tick cell cDNA library (prey) in sequential transformation procedure.

4. Select co-transformants after incubation at 30 °C for 5 days on synthetic defined (SD) minimal medium lacking tryptophan and leucine.

5. Pool all colonies from the plates and replated on SD minimal medium lacking tryptophan, leucine, histidine, and adenine.

6. Analyze positive clones for β-galactosidase activity by colony-filter assays [35].

7. Rescue the prey plasmids of positive clones using *E. coli* KC8.

8. Sequence the rescued prey plasmids using DNA sequencing using the SMART IV and CDS III primers using an Illumina MiSeq (Illumina, San Diego, CA, ISA).

9. Use the obtained sequences to identify candidate interacting proteins and protein interacting domains.

3.3 In Vitro Protein–Protein Interactions (Fig. 2)

3.3.1 Protein–Protein Pull-Down

1. For interactions, 2 μg of each protein are incubated for 2 h with shacking at room temperature (RT) in 200 μl of Mag c-Myc IP/Co-IP Buffer 1 supplied in the Pierce magnetic c-Myc-tag IP/Co-IP kit.

2. Magnetic beads are used for the protein pull-down using the Pierce magnetic c-Myc-tag IP/Co-IP kit. The used magnetic beads are specific for c-myc-tagged proteins, interacting with the protein of interest and pulling down the protein of interest-interacting protein (*see* **Note 2**). For the protein pull-down, wash 25 μl of magnetic beads with Mag c-Myc IP/Co-IP Buffer 1 following the manufacturer's recommendations (*see* **Note 3**).

3. After the 2 h incubation, add the sample containing the c-Myc-tagged protein to the pre-washed magnetic beads. Then, sample should be incubated for 30 min with shaking at RT. For negative control, incubate pre-washed magnetic beads with
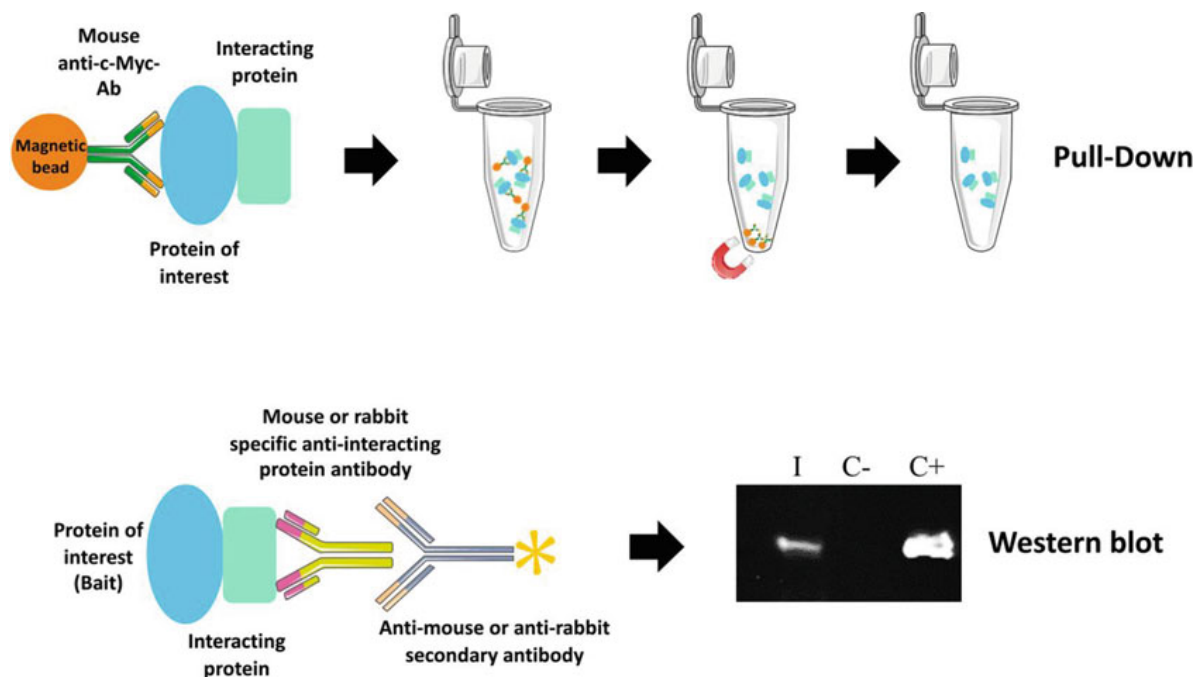
Fig. 2 Protein–protein interactions pull-down and corroboration by Western blot. Graphical representation of pull-down and Western blot steps for the corroboration of protein–protein interactions identified by Y2H. Graphical material has been obtained from free medical images at SMART-Servier Medical Art (https:/smart.servier.com/)

2 μg of protein of interest-interacting protein in 200 μl of Mag c-Myc IP/Co-IP Buffer 1 under the same conditions (*see* **Note 4**).

4. Remove unbound sample with a magnet to pull down the protein–protein complexes. Only the protein of interest should be attached to the magnetics beads through its c-Myc tag, as well as the proteins attached to the protein of interest.

5. Protein–protein complexes attached to the magnetic beads are washed with 300 μl of Mag c-Myc IP/Co-IP Buffer 2, diluted 1:20 in water. Samples are gently mixed, and supernatants are removed holding the protein–protein complexes with the magnet. Repeat this wash step twice.

6. Protein–protein complexes are washed with 300 μl of ultrapure water and gently mixed. Then, supernatants are removed as in **step 5**.

7. For sample elution from magnetic beads, add 30 μl of Laemli sample buffer with β-mercaptoethanol and vortex (*see* **Note 5**).

8. Collect samples with a gentle centrifugation and supernatants are saved, in new 1.5 ml Eppendorf tubes for Western blot analyses, holding the magnetic beads with the magnet.

1. Supernatants derived from protein–protein pull-down, negative controls and positive control recombinant protein are run and separated by electrophoresis in a 12% SDS polyacrylamide precast gel following standard procedures. Positive controls are the protein of interest-interacting protein because the western blot analysis is designed to detect the interacting protein of the protein–protein interactions to corroborate the interaction.

2. Proteins are transferred to a nitrocellulose blotting membrane for 1 h and 45 min at 200 V, 180 mA and 100 W in cold.

3. Then, the nitrocellulose membrane is blocked with 3% BSA blocking solution for 2 h at RT with shacking.

4. Membrane is washed with TBS-Tween wash buffer for 5 min at RT with shacking. Repeat this step three times.

5. Membrane is incubated with protein of interest-interacting protein specific primary antibody diluted in TBS overnight at 4 °C with shacking.

6. After incubation with primary antibody, membrane is washed three times as in **step 4**.

7. Goat anti-mouse or anti-rabbit immunoglobulin (IgG) (whole molecule) peroxidase antibody 1:1000 diluted in TBS with 3% BSA are used as secondary antibodies and incubated with membranes for 2 h at RT (*see* **Note 5**).

8. Wash membrane six times as in **steps 4** and **6**.

9. Immunoreactive proteins are visualized with chemiluminescence by incubating the membrane for 1 min with Pierce ECLWestern blotting substrate.

3.4 Musical Scores and Protein Interacting Domains (Fig. 3)

3.4.1 Epistemological Bases for a Musical Characterization

In recent years, various studies have called for the need to establish close cooperation between science and art [29, 36–39]. Some proposals [40] indicate how certain methodological and epistemological limitations can be overcome by the divergent perspectives of music and art, and set out their arguments on the philosophical reasons of Kant, Husserl, Heidegger, Wittgenstein, Schrödinger and Nishida Kitarō, based on the concept of intersubjectivity for the communication between scientific procedures and artistic and musical language. At the same time, the new musicology is proposed to have a place in the interdisciplinary scientific projects in order to promote concrete advances in the scientific, digital, psychological, and social fields [29, 41]. Recent studies [29] have shown that interdisciplinary research between musicology and molecular biology allows us to overcome the specific advances conducted by the exclusive methodological channel of science by implementing new musical and artistic perspectives in the analysis of the data. The musical translation of the protein–protein interactions answers a heterodox but scientifically based question, what
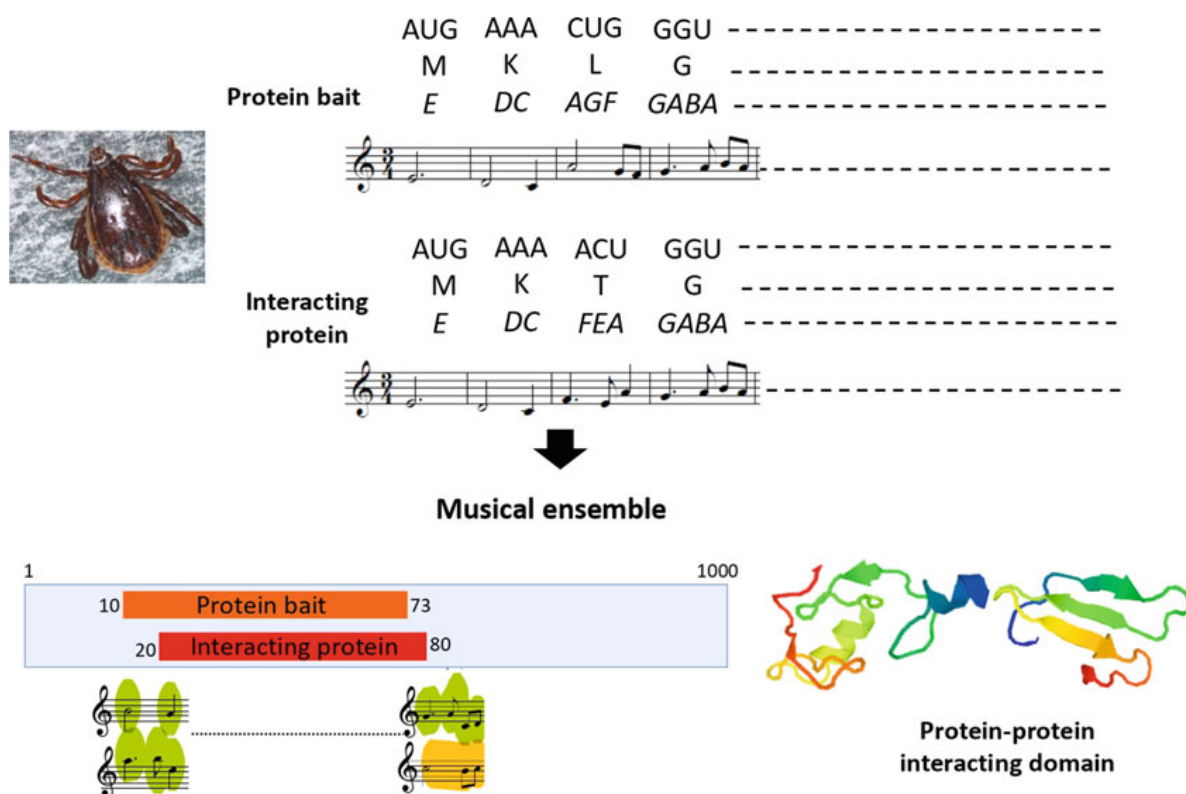
Fig. 3 The sound of protein–protein interactions. Musical ensembles of bait and interacting proteins predict and corroborate Y2H results of protein–protein interacting domains

kind of new research opportunities are opened up when we consider that the DNA sequence can be characterized and behaves sounding when transcribed in a musical language as an authentic work of musical nature, coherent in its formal, rhythmic and melodic configuration, and comparable through form, harmonic and counterpoint analysis with other sequences? The possible answer to this question is one of the bases for disarticulating the objective utopia and speaking, in interdisciplinary terms, of a topological objectivity promoted by the dialogue between science and art, seeking the principles of autonomy, singularity and creativity in science [42]. This cooperation seeks a fruitful metaphorical vision useful for scientific knowledge [43, 44] and makes music and its methods of analysis suitable cognitive instruments for other realities not specifically musical [45, 46].

3.4.2 Data Sounding and Musical Analysis

Following this line of research that approaches music closer to a scientific study [47–51], we have considered the most recent processes of "sonification" of data in the different scientific fields in order not to use them as aesthetic instruments that can facilitate the construction of musical works [52–54], but to make a coherent transcription susceptible in all its dimensions of a specific musical analysis following previously proposed algorithms [55–59]. The

perspective that musical analysis can provide on these transcriptions can not only confirm or corroborate the findings of the scientific methodology but, fundamentally, thanks to its creative and divergent nature, guide research into spaces opaque to the observation of the orthodox scientific method (*see* **Note 6**).

3.4.3 Determination of Pitches for each Amino Acid

1. The algorithm used to translate the DNA nucleotide coding sequences into pitches has been taken from previous research [44]. This algorithm facilitates the expression of the nitrogenous bases as a short sequence of pitches coded by arbitrary attribution on each of the steps of the diatonic scale (*see* **Note 7**). Each expression of the amino acid therefore has a unique and distinct pitch translation (Table 1). Therefore, the sequence of codons offers a line of tonal pitches whose reiterations, intervals, form, scope, tonal centers, inflection points and reiteration of sounds allow a congruent musical analysis.

2. In the algorithm used to translate the DNA coding sequences into pitches [44], use codons denoted as $X_n \, Y_n \, Z_n$ and musical notes as N_n. For every amino acid,

$$(aa)_{n1n2} = X_{\hat{O}} \, Y_{\hat{O}} \, Z_{\hat{O}} = N_{n2} \, N_{n1} = N_{n2} \, Y_{\hat{O}} \, Z_{\hat{O}} \, Z_{\tau}, \text{ for every } 1$$

$$\leq n1 \leq 6; 1 \leq n2 \leq 20; 1 \leq \alpha, \beta \, \tau \leq 4$$

   If by definition, aminoacid $(aa)_{n1n2} = X_\alpha \, Y_\alpha \, Z_\alpha = N_{n2}$, then if $n1 = 1$, $N_{n2} \, N_{n1} = N_{n2}$.

   The assignment of the musical note for the first codon for every aminoacid $(N_1, N_2, \ldots N_{20})$ is arbitrary and corresponded to the order given in Table 1.

3.4.4 Rhythmic Characterization

In order to represent melodic motifs, a sequence of pitches must be characterized by a rhythmic structure that allows them to be understood as a unit. Each codon is metrically equivalent to a bar and has a differentiated rhythmic and melodic character. The modified algorithm provides for each codon either a constant base of a note on which one or two more notes may follow, or a base of two musical notes followed by one or two more.

1. Provide a ternary structure (three beats of a quarter-note) and a binary subdivision measure (3/4), where the base of a single note occupies the entire measure (UGC = B, dotted-half-note, and the base of two notes is expressed as a half-plus-quarter-note (CAA = EF) (Table 1).

2. If the base is a single note accompanied by others, then chose to extend the base in the first two beats of the measure as half-note when it had the succession of a single note (UCG = GD half-plus-quarter-note) or two notes (UCC = GED, half-plus-two-eighth notes).

Table 1
Assignment of musical notes to different amino acids

Amino acid	Code	Codons	Musical notes	Metrics measure: 3/4 (3 beats)
Ala	A	GCA (aa)11 GCC (aa) 21 GCG (aa) 31 GCU (aa) 41	*CD* *CDED* *CDD* *CDA*	
Arg	R	CGA (aa) 12 CGC (aa) 22 CGG (aa) 32 CGU (aa) 42 AGA (aa) 52 AGG (aa) 62	*D* *DCD* *DGA* *DBA* *DFG* *DGA*	
Asn	N	AAC AAU	*DE* *DEBC*	
Asp	D	GAC GAU	*C* *CBC*	
Cys	C	UGC UGU	*B* *BBA*	
Gln	Q	CAA CAG	*EF* *EFD*	
Glu	E	GAA (aa) 17 GAG (aa) 27	*FG* *FGD*	
Gly	G	GGA GGC GGG GGU	*GA* *GACD* *GAGA* *GABA*	
His	H	CAC CAU	*AB* *ABE*	
Ile	I	AUA AUC AUU	*BC* *BCG* *BCF*	
Leu	L	CUA CUC CUG CUU UUA UUG	*A* *AG* *AGF* *AF* *AAG* *AGF*	
Lys	K	AAA AAG	*DC* *DCD*	
Met	M	AUG	*E*	
Phe	F	UUC UUU	*F* *FA*	

(continued)

Table 1
(continued)

Amino acid	Code	Codons	Musical notes	Metrics measure: 3/4 (3 beats)
Pro	P	CCA	*ED*	♩. ♪ ♫
		CCC	*EDED*	♩. ♪ ♩
		CCG	*EDD*	♩. ♪ ♩
		CCU	*EDA*	♩ ♫
Ser	S	UCA	*G*	♩ ♩
		UCC	*GED*	♩. ♩.
		UCG	*GD*	♩ ♫
		UCU	*GA*	♩ ♫
		AGC	*GCD*	
		AGU	*GBA*	♩ ♩
Thr	T	ACA	*FE*	♩. ♪ ♫
		ACC	*FEED*	♩. ♪ ♩
		ACG	*FED*	♩. ♪ ♩
		ACU	*FEA*	
Trp	W	AGG	*GF*	♩ ♩
Tyr	Y	UAC	*AG*	♩ ♩
		UAU	*AGBC*	♩. ♪ ♫
Val	V	GUA	*BA*	♩. ♪ ♩
		GUC	*BAG*	♩. ♪ ♫
		GUG	*BAGF*	♩. ♪ ♩
		GUU	*BAF*	

Musical notes follow the American standard pitch notation (ASPN) and the international pitch notation (IPN). Neo-latin translation for romance and other languages is C = do, D = re, E = mi, F = fa, G = sol, A = la, B = si

3. If the base is of two notes, establish a different metrical scheme for its expression: dotted-quarter-note-plus-eighth-note followed by a quarter-note (GCG = CDD), or by two eighth-notes (GCC = CDED).

4. When the algorithm provides the same melodic formula for a double base (GGA = SL; GGC = SLDR) and for a single base (UCA = S; UCU = SL), select the metric scheme half-plus-quarter-note in the first case and dotted-quarter-note-plus-dotted-quarter-note in the second case. In this way, each codon has a unique rhythmic and melodic definition.

5. Prepare all sound files and scores using the Finale (v. 2018) program (https://www.finalemusic.com).

3.4.5 Formal Analysis: Melodic, Harmonic, Formal and Counterpoint Study

1. Linear analysis. Once the pitches and rhythms have been defined (Table 1), the genetic sequence are read as a succession of rhythmic-melodic motifs that can configure larger units according to their own musical syntax: motif, semiphrase,

phrase, period, etc. This allows the analysis of the sequence from a formal perspective, which can be characterized in coherent musical units.

2. Replicability. The repetition of this transcription algorithm allows the comparison in strict musical terms between several sequences and species to provide for example (a) the predominance of certain melodic formulas in each species, (b) the fact that a single melodic form is never repeated more than three consecutive times, or (c) that all species show spaces with lower rhythmic movement [29].

3. Comparative analysis from a polyphonic perspective. In the same way, it is possible to implement a polyphonic perspective where genetic lines of different species can be seen as elements of a contrapuntal tissue that allows the observation, in a polyphonic context, of long sequences of unisons and melodic imitation effects even having long canonical structures that represent regions of amino acid homology between species. Use the creation of musical ensembles to support that orthologous proteins in different species are evolutionarily related and structurally conserved, and to predict and corroborate results of Y2H for protein–protein interactions [29].

3.5 Identification of Protective Epitopes or Immunological Quantum (Fig. 4)

3.5.1 Staining Protocol

1. Use standard glass slides ($3'' \times 1''$, $75.4\,mm \times 25.0\,mm \times 1\,mm$) with custom peptide content printed as spot duplicates (*see* **Note 8**).

2. Incubate the slide with the spot arrays with slight shaking (140 rpm) (*see* **Note 9**) for 30–60 min at RT in blocking buffer to reduce nonspecific interactions with the sample or secondary antibodies.

3. Wash $3\times$ 1 min at 140 rpm with standard buffer.

4. For equilibration, incubate the slide with slight shaking (140 rpm) for 15 min at RT in staining buffer.

5. Dilute the primary antibodies (IgGs) in staining buffer (*see* **Note 10**) and incubate with slight shaking (140 rpm) overnight at 2–8 °C with the primary sample diluted in staining buffer.

6. Wash $3\times$ 1 min at 140 rpm with standard buffer (*see* **Note 11**).

7. Dilute the secondary antibody Cy3 or Cy5 conjugated in staining buffer (*see* **Note 12**) and incubate with slight shaking (140 rpm) for 30 min at RT in the dark with the secondary antibody diluted in staining buffer.

8. Wash 3×1 min at 140 rpm with standard buffer and dip the slide two times into dipping buffer.

Identification of protective epitopes or immunological quantum
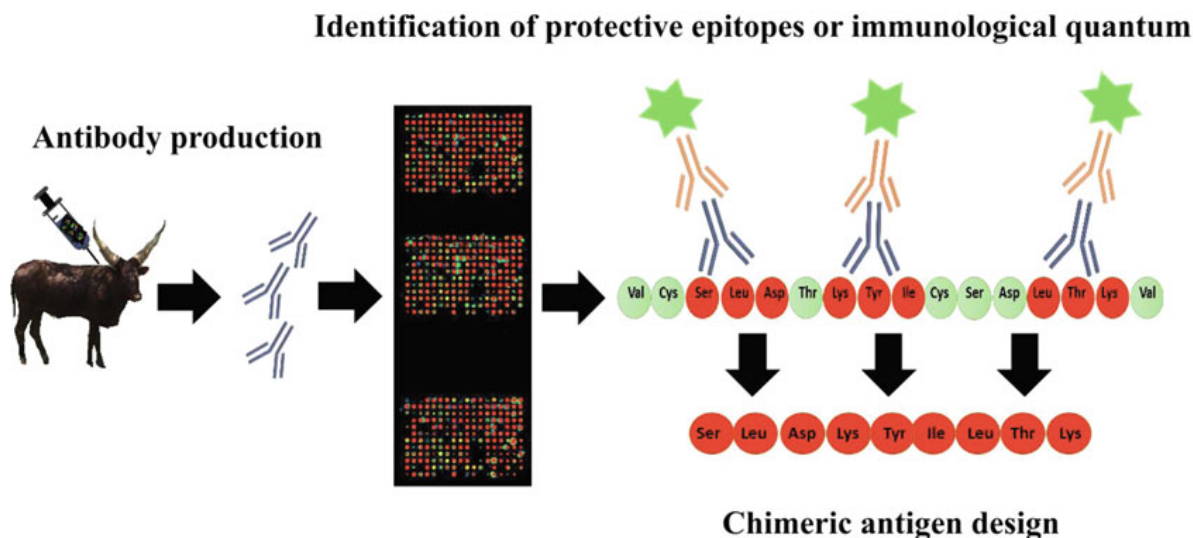
Fig. 4 Mapping of protective epitopes or immunological quantum. Identification of protective epitopes in protein interacting domains by antibodies from sera of cattle previously vaccinated with the recombinant target protein and used for the design and production of chimeric protective antigens

9. Dry the peptide microarray carefully in a stream of air and analyze the results in a microarray scanner (GenePix® 4100A) following the manual of the scanner for image recording.

3.5.2 Data Analysis

1. Use the provided data files for image analysis as well as with Excel spreadsheets with the microarray layout for manual spot annotation (*see* **Note 13**).

2. Perform the analysis as described in the PepSlide® Analyzer user manual (SICASYS).

3. Calculate for each spot of the array, the median and the mean of its raw, background, and foreground values. The interest are foreground values, which are computed by subtracting the background from the raw values.

4. Identify protective epitopes corresponding to higher foreground values.

4 Notes

1. Although Y2H could be performed as described or using other similar approaches, to improve the probability of identifying interacting proteins with high interaction confidence score it is possible to use Hybrigenics Services (Paris, France; www.hybrigenics-services.com) following previously described methods [29, 60, 61].

2. Use the Pierce magnetic c-Myc-tag IP/Co-IP kit if the target protein of interest has a c-Myc tag, but the

immunoprecipitation kit will depend on the tag of the protein of interest. It is important that the protein of interest-interacting protein does not have the same tag to avoid unspecific binding to the magnetic beads.

3. The number of magnetic beads will depend on the amount of protein sample used in the experiment and the binding capacity of the selected magnetic beads.

4. Incubate first the proteins to obtain the protein–protein complexes and then, add the magnetic beads to perform the binding of the c-Myc-tagged protein. Nevertheless, these steps can be performed the other way around by first coating the magnetic beads with the c-Myc-tagged protein and subsequently, adding the interacting protein to obtain the protein–protein complexes.

5. When the binding of the c-Myc-tagged protein to the magnetic beads is performed through a high-affinity mouse IgG monoclonal antibody attached to the beads that recognize the c-Myc-epitope tag, then in the elution step this antibody is also eluted together with the protein–protein complexes. Consequently, when using an anti-mouse secondary antibody in the experiment, the anti c-Myc antibody will be identified in **step 9** of the western blot analysis.

6. This method of sonification will only take into account the sound parameters of pitch and rhythm. A more complex analysis can be done by translating other characteristics of the DNA code to the timbre and dynamics parameters.

7. The diatonic scale is only one of the many possible scales, all of which are products of a cultural construct. Diatonism facilitates an approach to the analysis of tonal and modal nature. Possible codifications with different scales such as chromatic, octatonic, modal scales, among other offer new perspectives for alternative sonifications.

8. Glass slides with custom peptides or for the identification of protective epitopes could be performed as described using PEPperPRINT Services (Heidelberg, Germany; https://www.pepperprint.com/technology/peptide-microarray-analysis).

9. Shaking incubation significantly improves sample circulation to avoid gradients that may cause a bias in microarray data. Therefore, we recommend an orbital shaker at 140 rpm. Rocking incubation, however, may cause dewetting of the microarray surface and should be avoided [62].

10. Depending on the nature of the sample and the interaction parameters, serum and plasma dilutions may vary from 1:10 to 1:10000 with 1:1000 as recommended starting dilution. For

purified antibodies, starting concentration of 1 μg/ml is recommended.

11. Washing times and repeats have to be adjusted with low affinity interactions and/or high off-rates. Vigorous washing may release antibodies and other proteins from the peptides and thus cause a loss in primary signals.

12. Depending on the nature of primary and secondary antibodies, the dilution may vary from 1:500 to 1:10000. Please note that higher concentrations can cause stronger background signals.

13. Please note that morphologies are rectangular, which may require certain adjustments of spot geometry settings.

Acknowledgments

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Chapter 18

Screening for the "Achilles Heel" of Hyalomma anatolicum Ticks by RNA Interference Technology and an Update on Anti-Tick Vaccine Design

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Abstract

Over the years, RNA interference (RNAi) has evolved as a valuable tool to study the tick gene function, screening and preliminary characterization of tick-protective antigens in a relatively short time, with a minimal use of laboratory animals before conducting expensive vaccine trials for the development of improved vaccine composition. In this process, a double-stranded RNA (dsRNA) of gene of interest is introduced into the tick system which specifically suppresses expression of a target gene. The results of RNAi-based gene silencing were interpreted by reduction in targeted gene transcript, changes in phenotypic data and anatomical/ biochemical changes in ticks; thereby, providing a clue to the probable role played by the gene in the tick biological system. Across the globe, various tick research groups applied RNAi technique for characterization and identification of new anti-tick vaccine targets. Herein, we used the RNAi tool in *Hyalomma anatolicum* ticks for identification and characterization of vaccine candidates.

Key words *Hyalomma anatolicum*, RNA interference, Vaccine

1 Introduction

Biologists can switch off a specific gene in a variety of organisms in order to deduce gene functions with the help of posttranscriptional gene silencing (PTGS). The siRNA can be generated corresponding to the gene under study, followed by identification of phenotypic changes after transfection. These phenotypic changes can be in terms of viability or biochemical changes; thereby, providing a clue to the probable role played by the gene. RNA interference (RNAi), a well-established molecular technique has been applied in many organisms including ticks [1]. In this technique, double-stranded RNA (dsRNA) is used which specifically suppresses expression of a target gene through small interfering RNA (siRNA) pathways. It has been shown to be a valuable tool for the study of tick gene function, the characterization of tick

pathogen interface, the screening and characterization of tick-protective antigens [2]. The minimum size of dsRNA recommended for RNAi is ~200 bp [3]. The RNAi experiment in non-mammalian targets can be performed with dsRNA of 400 bp or larger [4, 5]. It is suggested that longer dsRNA molecules are more effective on a molar basis of silencing protein expression, but a higher concentration of smaller dsRNA molecules may also have similar silencing effect [3]. There are four different methods of delivery of dsRNAs in tick viz., (1) injection, (2) soaking, (3) feeding, and (4) virus production of dsRNA [6]. The result of RNAi based gene silencing has been interpreted differently. First, reduction in targeted gene transcript which is measured by quantitative PCR; second, changes in phenotypic data such as tick survivability, engorgement weight, oviposition [7–10]; third, pathological/anatomical changes; and forth, biochemical changes.

The first challenge in the development of anti-tick vaccine development is the identification of novel antigen(s) which should be recognizable by the host immune system and conserved across the tick species as vaccine for individual tick species is not a suitable option in field situation. Through RNAi, it is possible to screen a large number of genes to identify potential vaccine candidate in a relatively short time with a minimal use of laboratory animals. Selected antigen could then be characterized and evaluated as recombinant proteins in controlled vaccine trials [2]. The schematic representation of selection of vaccine candidates through RNAi technology is illustrated in Fig. 1. Here, we have used three target candidates which are highly conserved between different tick species viz., (1) Ferritin-2 (FER2)—gut specific protein secreted into the tick hemolymph and acts as an iron transporter [11]. (2) Tropomysin (TPM)—allergenic, actin-binding protein present in all muscle and non-muscle cells that regulates the actin organization [12]. (3) Subolesin (SUB)—tick-protective intracellular protein and structural and functional orthologue of insect and vertebrate akirins. It is evolutionary conserved in nature and function as transcription factors in the regulation of gene expression and thus affecting multiple cellular processes such as the innate immune response, digestion, reproduction, and development [13].

2   Materials

All the reagents for the preparation of buffers should be of analytical grade. The buffers are to be prepared using deionized double distilled water or Milli-Q water and to be stored at 4 °C (unless otherwise indicated). For RNA work, plasticwares are to be pre-treated with 0.1% diethylpyrocorbonate (DEPC) for 12 h, at 37 °C and subsequently sterilized by autoclaving or nuclease free plasticwares are preferred. All animal experimentations need to be conducted following the standard procedure and after prior approval from the competent authority.
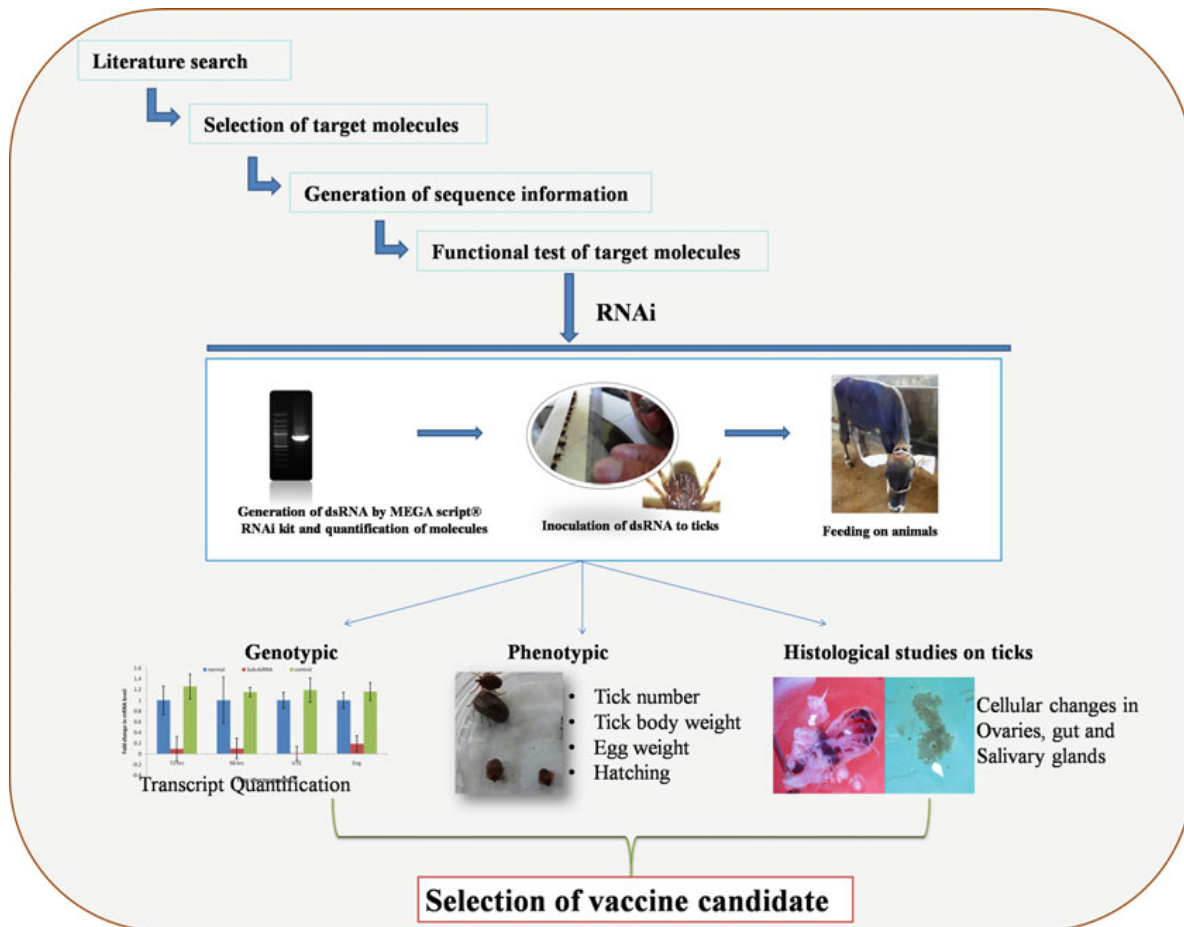
Fig. 1 Schematic diagram showing the process of selection of a vaccine candidate through RNAi technology

2.1 Chemicals and Reagents

Standard chemicals and reagents are important requirement for successful experimentation in molecular biology. Following chemicals and reagents may be used for isolation of RNA from ticks. Trizol® reagent (Invitrogen, USA), Chloroform, Isoamyl alcohol, Absolute ethanol, Nuclease free water, Diethylpyrocarbonate, RNA later, RNase Zap® (Ambion, USA).

For gene amplification and cloning, following chemicals and reagents may be used. Isolated RNA samples, ReverseAid H minus Reverse Transcriptase, RiboLock RNase Inhibitor, dNTPs, Oligo (dT), 5× RT buffer, DreamTaq DNA polymerase, 10×DreamTaq Green Buffer, 100 bp plus DNA ladder, GeneJET gel extraction kit, InsTAclone PCR cloning kit, ampicillin sodium, X-Gal RTU, IPTG and 6× DNA loading dye from Thermo Scientific, USA. 0.5 M EDTA, Isoamyl alcohol, LB Broth Miller (Luria-Bertani), and LB Agar Miller (Luria-Bertani). Nuclease free water, PCR primers (IDT, USA), *Escherichia coli*-DH5α strain (Invitrogen, USA), agarose, Tris (Hydroxymethyl) aminomethane, acetic acid, ethidium bromide, absolute ethanol.

Gene silencing experiments can be done in tick through RNAi technique where following chemicals and reagents are required:

pGEM-*luc* DNA (Promega, USA), Ampicillin sodium, DreamTaq DNA polymerase, dNTPs, 100 bp plus DNA ladder and Gene JET Gel Extraction Kit from Thermo Scientific, USA. 0.5 M EDTA, Tris-EDTA Buffer pH 8.0, Hydrogen Peroxide. MEGAscript® RNAi Kit and Nuclease free water from Ambion, USA. Tris (Hydroxymethyl) aminomethane and Acetone. Absolute ethanol, Acetic acid, Ethidium bromide, Agarose, QIAprep® Spin Miniprep Kit (Qiagen, Germany), PCR Primers (IDT, USA).

2.2 Buffers

1. DEPC treated water: Dissolve 1 ml Diethylpyrocarbonate (DEPC) in 999 ml of distilled water using magnetic stirrer or constant shaking for 4–6 h. The DEPC treated water is used for removing RNAses from plasticwares and glasswares for RNA isolation.

2. 70% ethanol: Mix 70 ml of absolute ethanol with 30 ml of distilled water.

3. PCR primer stock (100 mM): Add 100 times NFW or TE buffer to the molar concentration of primer. Keep the stock at 4 °C for 4–8 h and store at −20 °C. All the used primers are listed in Table 1

4. 50× TAE buffer: Add 121.14 g of Tris–HCl, 50 ml 0.5 M EDTA (pH 5.0), 28.8 ml of glacial acetic acid and make up the volume up to 500 ml with distilled water.

5. 1% agarose: Add 250 mg of agarose to 25 ml of 1× TAE buffer, boil the solution and cool down to 50–55 °C, add 1 μl ethidium bromide (10 mg/ml) and pour in gel casting tray (*see* **Note 1**).

2.3 Reference Biological Material

The RNAi experiment for gene silencing study can be done on any stage of the tick viz. eggs, larvae, nymphs and adults [2], but the adult ticks are considered the most suitable and easy for experimentation and data recording. To get good number of homogenous colony of adult ticks, *H. anatolicum* can be maintained on laboratory animals under laboratory conditions [14]. In our experiment, the *H. anatolicum* Izatnagar isolate (NBAII/IVRI/HA/1/1998) maintained in the Entomology laboratory, Division of Parasitology of the institute are used. The fully engorged *H. anatolicum* ticks are thoroughly rinsed with distilled water, placed on filter paper kept inside the tick rearing glass tubes. The glass tubes are covered with muslin cloth and kept at 28 °C temperature and 85% relative humidity (RH) for 7–10 days for oviposition. Care is to be taken for removing dead ticks to avoid fungal growth and contamination of eggs. Normally, 15–21 days are required for hatching of larvae. Approximately, 200 larvae or larvae hatched from 20 mg eggs are released on ear pinna of New Zealand white rabbits for feeding. The engorged nymphs are to be collected from ear bags, rinsed

Table 1
Primers for amplification and dsRNA generation of Ferritin 2 (FER2), Tropomyosin (TPM), Subolesin (SUB) genes of Hyalomma anatolicum and RNAi control gene Luciferase (LUC)

Oligos name	Primer set	Nature	Use
FER2	5′CGCAGCCACCATGTTTCGAATTGTAG 3′ 5′CTAGGTGCGCAGTTGCTGGTCCAG 3′		For amplification, sequencing and gene conservation study
TPM	5′ ATGGAKGCCATCAAGAARAARATGCAG 3′ 5′ GAGCAGCGGGTRGAAACAACGGC 3′	Degenerate primers	
SUB	5′ATGGCTTGTGCGACATTAAAGC 3′ 5′TTACGACAAATAGCTGGGCGT 3′		
FER2	5′TAATACGACTCACTATAGGGCCTTGTCCCCACCAGCA 3′ 5′TAATACGACTCACTATAGGGACGACAAATAGCTGGGCG 3′	Primers with T7 promoter (highlighted seq)	For dsRNA generation (RNAi)
TPM	5′TAATACGACTCACTATAGGGCCGCAAAGTTCGAGCCGT 3′ 5′TAATACGACTCACTATAGGGCCGGACTTGACCTGCCACA 3′	pGE-luc Vector Sequence	
SUB	5′TAATACGACTCACTATAGGGCCTTGTCCCCACCAGCA 3′ 5′TAATACGACTCACTATAGGGACGACAAATAGCTGGGCG 3′		
LUC	5′TAATACGACTCACTATAGGGCCGGCGAACGACATTTA3′ 5′TAATACGACTCACTATAGGGCCCGGTATCCAGATCCAC3′		

with distilled water and to be kept at 28 °C temperature and 85% RH for hatching. The freshly hatched adults are to be kept unfed for 7–10 days for further use in gene silencing experiments.

2.4 Experimental Animals

New Zealand white rabbits (1–1.5 year old) and crossbred male calves (>6 month old) (*Bos indicus* × *B. taurus*) are to be maintained in the tick-proof shed with *ab libitum* feed and water. Prior to conducting experiments on animals, necessary permission from regulatory authority is required.

3 Methods

3.1 Isolation of Total RNA and cDNA Synthesis

1. Utmost care is to be taken while isolating RNA to prevent RNA degradation by RNAses. Prior to RNA isolation, the equipment and work station are to be wiped with RNase Zap® to decontaminate them from RNase. Nuclease free plasticwares, micropipette tips having aerosol barrier are to be used.

2. For extraction of total RNA, unfed adults weighing about 50–100 mg are preferred. Take the tick sample in microfuge tube and place in liquid nitrogen for 5 min or overnight at −80 °C. Homogenize the frozen tick sample using DEPC treated mortar and pestle in 1 ml of Trizol® and incubate for 30 min at 4 °C or overnight at −20 °C to permit complete dissociation of nucleoprotein complex.

3. Centrifuge the homogenate at $15,000 \times g$ for 10 min at 4 °C and transfer the supernatant carefully to another microfuge tube using pipette. To the supernatant, add 0.2 volume of chloroform per 1 ml of Trizol® and shake vigorously for 15–20 s, incubate at room temperature for 5 min. Centrifuge the mixture@$11,000 \times g$ for 15 min at 4 °C.

4. Transfer the aqueous phase carefully to a microcentrifuge tube and precipitate the RNA by mixing with chilled isopropyl alcohol (0.5 ml per ml of Trizol® reagent). Incubate the mixture at 4 °C for 10 min or −20 °C for overnight and centrifuge@$11,000 \times g$ for 15 min at 4 °C (Generally, precipitated RNA is often invisible in the form of gel like pellet at the bottom of the tube before centrifugation). Discard the supernatant and wash the pellet thrice with 500 μl of chilled 70% ethanol (each time). Every time, gently vortex the tube and centrifuge@$11,000 \times g$ for 2 min at 4 °C and discard the ethanol.

5. Finally, the RNA pellet is to be air dried and dissolved in 50–100 μl of NFW and stored at −80 °C in 10 μl aliquot (to prevent degradation during freezing-thawing). Run the agarose gel electrophoresis to check the integrity of the RNA

and determine the RNA concentration by NanoDrop Spectro-photometer or Qubit® Fluorometer or any other way.

6. Use the isolated total RNA for cDNA synthesis using Oligo (dT) primers as per the standard protocol [15].

Example: A 20 μl reverse transcription reaction protocol is as follows, Initially, incubate the mixture of template RNA-5 μl (4 μg) along with 1 μl Oligo (dT) primer (0.5 μg) at 70 °C for 5 min, snap cool on ice and then add Reverse transcriptase buffer (5×)- 4 μl, RNase inhibitor (40 U/μl)- 0.5 μl, dNTP mix (10 mM)- 2 μl, make the volume to 19 μl by adding distilled water, mix it properly and incubate at 37 °C for 5 min. Finally, add 1 μl Mu-MLV RT-H Minus (200 U/μl) to the reaction and incubate at 42 °C for 1 h for first strand cDNA synthesis and inactivate the reverse transcriptase by incubating the reaction mixture at 70 °C for 10 min. Store the prepared cDNA at −20 °C until used.

3.2 Primer Design and Synthesis

To design the primers, retrieve the available respective gene sequences available in public domain (GenBank- NCBI). Align the retrieved nucleotide as well as amino acid sequences from various ticks and arthropods using DNA STAR (Laser Gene Software). Select the conserved regions among different tick gene sequences to design PCR primers using bioinformatics tools like GeneTool (BTI software), DNA STAR (Laser Gene Software) and online software NCBI-BLAST to check the designed primers. The synthesized primers in lyophilized form are to be suspended in NFW (100 times of nmoles quantity) to get 100 μM concentration. Dilute the stock solution (ten times) in NFW to obtain a working solution of 10 μM (10 pmol/μl). Store the stock solution at −20 °C and working solution at 4 °C.

3.3 Amplification of FER2, TPM, and SUB Genes of H. anatolicum

1. Calculate the melting temperature (T_m) of primers designed for each gene based on the formulae, $2(A + T) + 4(G + C)$.

2. Run the gradient PCR at different annealing temperatures based upon calculated melting temperature to optimize the annealing temperature in PCR.

3. Prepare the PCR mixture in 25 μl reaction volume containing 70–100 ng of cDNA, 10 pmol of each forward and reverse primers of respective genes (Table 1), 1.5 mM $MgCl_2$, 200 μM of each dNTPs, and 1 U of True start HotstartTaq DNA polymerase and use a thermal cycler with a preheated lid for amplification process.

4. The cycling conditions for the above mentioned genes can be set as: initial denaturation of strands for 5 min at 95 °C, followed by 35 repeat cycles of denaturation at 95 °C for 30 s, annealing of primers at 58 °C for 45 s (FER2), 56 °C for 50 s

(SUB), 60 °C for 60 s (TPM) and extension of strands at 72 °C for 60 s. Final extension of the synthesized strands are done at 72 °C for 10 min.

5. Confirm the amplification by running the product on an ethidium bromide stained 1% agarose gel and visualize the amplicon on a transilluminator under UV light.

3.4 Cloning of FER2, TPM & SUB Genes of H. anatolicum in pTZ57R/T Vector System

1. Use commercially available purification kits to reduce the time for purifications of desired PCR products. Gene JET®GelExtraction Kit (MBI Fermentas, USA) was used for the purification of the desired PCR products of FER2, TPM & SUB genes from *H. anatolicum*. Carry out 1% agarose gel electrophoresis by loading 100 μl of the PCR product for each gene to get the good yield of desired products. After visualizing under long range UV light, excise the desired band with minimum time exposure and transfer to a sterile 1.5 ml centrifuge tube and elute in 50 μl nuclease free water.

2. Use InsTAclone PCR cloning kit (MBI Fermentas, USA) which contained cloning vector (pTZ57R/T), T4 ligase, 5× ligation buffer and reagents for competent cell preparation like c-media, T sol-A and T sol-B for the ligation and cloning of desired PCR products of genes according to the manufacturer's protocol. A standard 30 μl ligation reaction is to be set in a 0.2 ml tube on ice to ligate the purified PCR product (5–10 μl product depending on concentration) into pTZ57R/T cloning vector (0.18 pmole ends) 3 μl, in the presence of 6 μl of 5× ligation buffer, 1 μl T4 DNA ligase and the volume is to be made up to 30 μl using nuclease free water. Mix all the reagents well, spin and incubate at 4 °C, overnight. Keep the ligated product at −20 °C if transformation is not done on the same day.

3. To prepare the *Escherichia coli* DH5α competent cells, day before the transformation inoculate 200 μl culture of *E. coli* DH5α cells in 2 ml of c-media in 15 ml sterile tube for overnight in orbital shaker maintained at 37 °C, 180 rpm.

4. On the day of transformation, fresh culture is to be initiated in a sterile 15 ml tube by inoculating 1/10th of overnight cultured cells into a pre-warmed (37 °C) c-media (for two transformation 1.5 ml c-media and 150 μl overnight cultured cells) for 20–30 min at 180 rpm.

5. The freshly grown culture is to be transferred into a pre-chilled 1.5 ml sterile tube kept on ice. Subsequently, harvest cells by centrifugation at 3500 × *g* for 2 min at 4 °C. Meanwhile, T-solution is to be prepared by mixing the equal volume of T sol-A and T sol-B (for 1.5 ml culture 350 μl T-solution was required) and keep on ice.

6. Resuspend the bacterial pellet in 250 μl of T-solution and incubate on ice for 5 min. Again, centrifuge the cell suspension for 1 min at 3500 × *g*, 4 °C to get bacterial pellet. Discard the supernatant, mix the pellet again in rest of the T-solution (i.e., 100 μl) and keep on ice.

7. Before the start of competent cells preparation, Luria-Bertani (LB) agar plates are to be prepared by adding 2 g of LB agar in a 100 ml cleaned flask containing 50 ml of distilled water (40 g/ l). The mixture is to be autoclaved at 121 °C and 15 lb. for 30 min. When agar media is cooled to 50–55 °C, 50 μl ampicillin (100 mg/ml), 100 μl X-gal (20 mg/ml) and 25 μl 1 M IPTG are to be added (according to cloning vector), mixed and poured into two sterile culture plates (20–25 ml for one plate) keep under laminar air flow cabinet for solidification.

8. To transform the cloned vector into bacterial cells, include 2–5 μl of ligated product into a freshly prepared 50 μl of competent cells, incubate on ice for 5 min. Transfer the mixture on pre-warmed (37 °C) LB agar plate and spread it using L-shaped spreader and allowed to dry under laminar flow. Wrap the plate in aluminum foil and incubate at 37 °C for 12–16 h in inverted position for the development of recombinant colonies.

9. Screen the recombinant clones using blue-white screening method (α-complementation). Pick the white colonies and confirmation of the insert in the vector is to be carried out through colony PCR. Briefly, pick the single white colony and transfer to a sterile 2 ml micro centrifuge tube containing 500 μl autoclaved LB broth with 100 μg/ml ampicillin. Allow to grow the cells for 3–4 h at 37 °C and 180 rpm. Boil one in ten dilution of the recombinant *E. coli* culture in NFW for 10 min and centrifuge at 9500 × *g* for 5 min. Use the 2 μl supernatant as template in colony PCR using gene-specific forward and reverse primers for confirmation of recombinants.

10. Confirm the presence of insert in the plasmid through DNA sequencing.

3.5 Plasmid Isolation from Positive Clones for Double-Stranded RNA Synthesis

1. Isolate the plasmid DNA from positive recombinant colonies by alkaline lysis method [16]. After confirmation by colony PCR, inoculate the positive single bacterial colony to the 5 ml LB medium containing 100 μg/ml ampicillin for 12 h at 37 °C in shaker incubator. Use the fresh culture in log phase for plasmid DNA (pTZ57R/T-FER2, pTZ57R/T-TPM and pTZ57R/T-SUB) isolation.

2. Centrifuge the bacterial culture (5 ml) at 9500 × *g* for 10 min and resuspend the cell pellet in 100 μl ice cold solution I (20 mM glucose, 25 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0).

3. Add 100 µl of solution II (0.2 N NaOH, 1% SDS) to the resuspended cells and keep for 5 min to lyse the cells.

4. Add 150 µl of solution III (5 mM potassium acetate in glacial acetic acid) to the cells and keep at 4 °C for 10 min followed by centrifugation at 9500 × g for 10 min to remove the bacterial debris and chromosomal DNA by precipitation reaction.

5. Treat the supernatant with equal volume of phenol: chloroform (1:1 v/v) and separate the aqueous phase to another tube.

6. Precipitate the plasmid DNA from the aqueous phase with two volumes of ethanol in 0.2 M sodium chloride, wash in 70% ethanol, air dry and resuspend in nuclease free water. Measure the concentration of plasmid DNA by spectrophotometer and store at −20 °C for next steps.

3.6 Primer Designing for RNAi

Design the gene-specific primers and unrelated gene *Luc* to get more than 400 bp amplified products by adding consensus T7 promoter sequence at 5′ end of both the forward and reverse primer sets (Table 1). The T7 promoter sequence is: 5′ TAA TAC GAC TCA CTA TAG GG 3′.

3.7 Generation of dsRNA

1. A good amount of purified target gene fragments having T7 promoter sequence at 5′ ends is required. Use the gene-specific positive plasmid viz. pTZ57R/T-FER2, pTZ57R/T-TPM and pTZ57R/T-SUB and luciferase gene from vector pGEM-luc (Promega, USA) as template and specifically designed primers containing T7 promoter at 5′ end in a PCR. For each gene, set a 25 µl reaction in a 0.2 ml PCR tube containing 10× Dream-Taq Green Buffer (Contain 20 mM MgCl$_2$), dNTPs (10 mM each), DreamTaq DNA Polymerase (5 µ/µl), forward and reverse primers (10 µM each) and gene-specific plasmid (20 ng) as template in each reaction. Make up the final volume with NFW (*see* **Note 2**). The cycling conditions for amplification of each gene are as follows:

Parameters	For FER2/SUB/LUC		For TPM		Cycle
---	Temp.	Time	Temp.	Time	
Step 1					
Initial denaturation	95 °C	3 min	95 °C	3 min	1
Denaturation	94 °C	40 s	94 °C	40 s	5
Annealing	56 °C	30 s	55 °C	30 s	
Extension	72 °C	40 s	72 °C	50 s	
Step 2					
Denaturation	94 °C	40 s	94 °C	40 s	32

(continued)

Parameters	For FER2/SUB/LUC		For TPM		Cycle
	Temp.	Time	Temp.	Time	
Annealing	60 °C	30 s	60 °C	30 s	
Extension	72 °C	40 s	72 °C	50 s	
Final extension	72 °C	15 min	72 °C	15 min	1

2. Check the amplified products by electrophoresis in 1% agarose gel and view under UV transilluminator. Use the Gene JET® Gel Extraction Kit (MBI Fermentas, USA) for the purification of the desired PCR products of FER2, TPM & SUB genes of *H. anatolicum* as described earlier (Subheading 3.4) to get millions of copy of specific target gene fragments.

3. For *in vitro* transcription, use the commercially available *in vitro* transcription kit for better yield. In the current method, the MEGAscript® RNAi Kit (Ambion, USA) is used for *in vitro* transcription and purification of dsRNA as per the manufacturer's protocol with slight modifications. Initially, prepare the reaction mixture consisting of all the four types of nucleotides, purified PCR product as template, T7 DNA dependent RNA polymerase along with 10× T7 buffer. Mix the solution properly and make up the volume to 20 μl with NFW. Spin the reaction mixture and incubate at 37 °C for 15 h followed by heat inactivation of enzyme and denature the nucleic acid at 75 °C for 5 min. Further, for conversion of ssRNA to dsRNA, reduce the reaction temperature slowly for a period of 2–4 h to reach the room temperature. Confirm the dsRNA formation by running on 1% agarose gel by loading the 1:50 diluted dsRNA solution. Upon confirmation, subject the dsRNA to DNase-1 and RNase treatment.

4. Conduct the nuclease digestion reaction for dsRNA solution to remove excess template DNA and any unstable ssRNA in the solution. For each 50 μl reaction set, add RNase (1 μl), DNase 1 (4 U), 10× digestion buffer (5 μl) in 0.2 ml tube and make up the volume to 50 μl by adding NFW. Mix and spin the reaction components and incubate at 37 °C for 60–90 min in incubator to complete the process.

5. After treatment of dsRNA with DNase-I and RNase, to remove the impurities like proteins, free nucleotides and nucleic acid degraded products, mix the dsRNA solution with 10× binding buffer and absolute ethanol. Load the reaction mixture in to the filter cartridge, and centrifuge at 18,879 × g for 2 min at room temperature.

6. Pass the wash buffer (500 μl) through the filter cartridge twice by centrifugation at 18,879 × *g* for 2 min each to wash the dsRNA.

7. Later, discard the flow-through from collection tube and spin the empty filter cartridge at the same speed for 30 s to remove any traces of liquid.

8. Elute the dsRNA from filter cartridge by adding 80–100 μl of preheated (60–65 °C) elution buffer (TE buffer, 10 mM Tris–HCl pH 7, 1 mM EDTA) at the center of cartridge, keep it for 2 min and centrifuge at 18,879 × *g* for 2 min.

9. Check the integrity and efficiency of duplex formation of dsRNA by resolving in 1% agarose gel and by NanoDrop spectrophotometer (5 μl sample in 1:20 dilution), respectively. Quantify the purified dsRNA by measuring the absorbance at 260 nm. Dilute the dsRNA sample at 1:10 in elution buffer (1 μl sample + 9 μl elution buffer) and read the absorbance in a NanoDrop® spectrophotometer.

10. Calculate the concentration of RNA in μg/ml using the formulae A_{260} X dilution factor X 40 =μg/ml RNA.

11. Determine the molecular weight of dsRNA by submitting the dsRNA sequence to a web-based software Oligo Calc (www.basic.northwestern.   edu/biotools/oligocalc.html/#helpMW). The formula is Molecular weight of a molecule = 1 mole = 6.023×10^{23} molecules (Avogadro's number). Ex. If molecular weight of dsRNA is Z gram and concentration of dsRNA in a sample is Υ ng/μl. The number of dsRNA molecules present in per microliter of sample is equal to,

$$\frac{\Upsilon}{Z} \times 6.023 \times 10^{14}$$

***3.8 Injection of dsRNA into the Unfed Adult of* H. anatolicum**

1. Before starting dsRNA injection, separate the unfed male and female adult ticks under stereo-zoom microscope based on their scutum and other key features. Keep the adult male ticks in BOD incubator maintained at 28 °C. At a time, select a batch of 25–30 female ticks (7–10 days old) for inoculation of gene of interest. To avoid the contamination, wash the batch of selected female ticks in serial manner by distilled water, 3% hydrogen peroxide, distilled water, 70% ethanol and finally by excess of distilled water. Remove the excess moisture by soaking the ticks on paper towel. Keep 10 μl Hamiltion® syringe, 34 G, 12.5 mm length custom designed RN needle ready before injection by cleaning with distilled water followed by acetone and again distilled water for 5–6 times.

2. Fix the labeling tape (Tarson, India) on one side of clean plastic scale. At a time, choose a group of 10 female ticks and adhere dorsally on side by side to the tape. To restrict the movement of
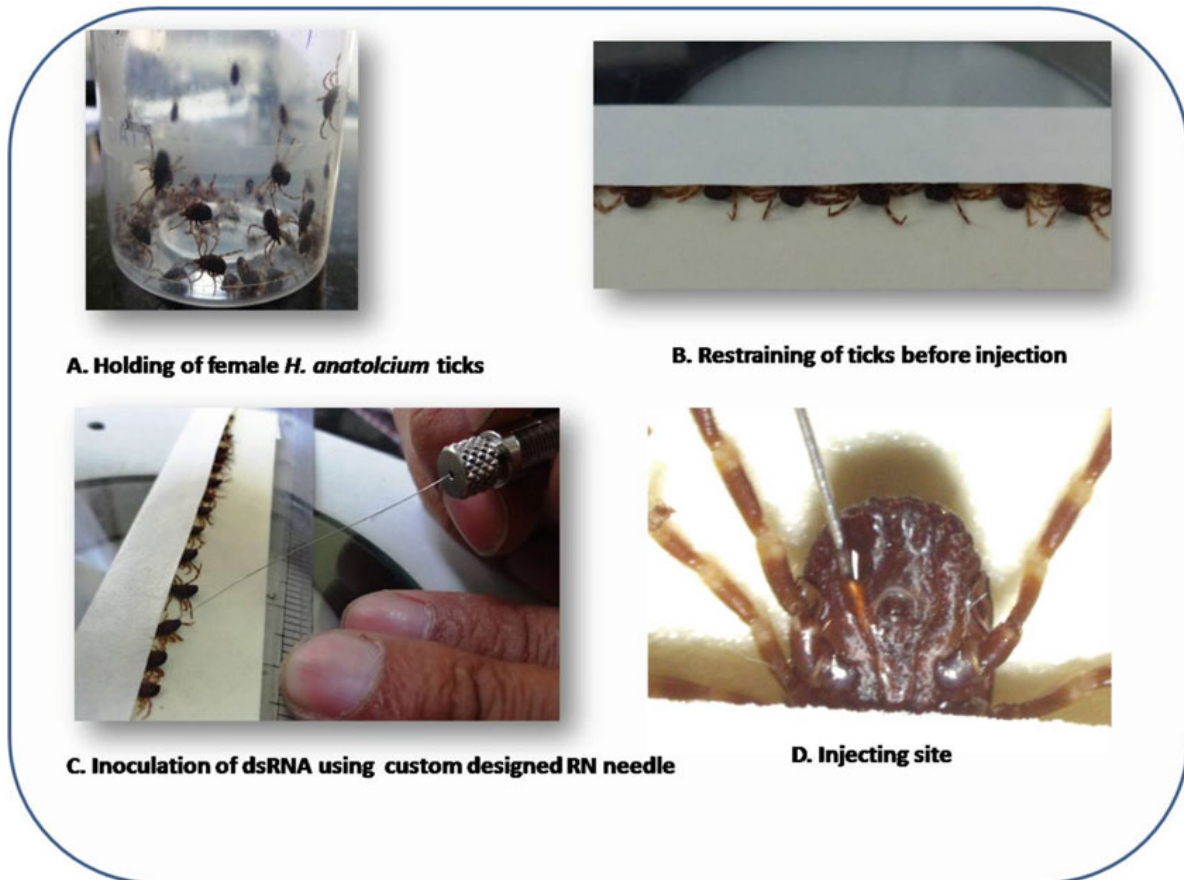
Fig. 2 Overview of steps involved in dsRNA injection

tick while adhering, slightly provide thermal shock by keeping ticks in 4 °C for 5 min. Secure the ticks by placing another piece of tape on above the anterior half body part of the fixed ticks (*see* **Note 3**).

3. Prior to injection, puncture the tick surface at fourth coxae posterior-ventral region by 30 G insulin needle for easy inoculation using 34 G RN needle. Inoculate individual tick with 0.5–1 μl of dsRNA of gene of interest, diluted in elution buffer (10 mM Tris–HCl, pH 7 and 1 mM EDTA) having concentration of 5×10^{10} to 5×10^{11} molecules/μl (Fig. 2).

4. Similar to the gene of interest, inoculate the group of ticks with dsRNA of control gene, i.e., luciferase gene amplified from vector pGEM-*luc* (Promega, USA) as mentioned for comparative analysis. After injection, gently remove the individual tick with the help of fine forceps capturing the legs from the adhesive tape and allow to move in 100 ml wide bottom container to check the biological activity of the ticks (*see* **Note 4**).

5. Incubate the ticks in BOD incubator at 85% relative humidity (RH) and 28 °C temperature for 24 h.

Assess the vigor and activity of the inoculated and control ticks by visual observation and critical examination before release of equal number of male and female ticks on ear pinna of calf using tick feeding bag.

3.9 Evaluation of RNAi Effects

3.9.1 Evaluation of RNAi Effects on Biological Activity

1. Select the cross bred male calves (>6 month age) for feeding of gene silenced adult ticks. Trim the hairs of ear and its surrounding area to facilitate attachment of ticks.

2. Release batch of 25–30 injected female ticks along with equal number of males on each ear and tie the bag firmly to prevent escape of ticks.

3. After 24 h of release of ticks, check the bag for any damage. Check the ear bags after 48 h to collect any dead or unattached ticks. Check the tick feeding bag everyday and collect the fully engorged ticks, count the number and weigh. At the end of feeding experiment, on tenth day, collect all female ticks from animals which are designated as unable to engorge (UTE) tick.

4. Evaluate the effects of gene silencing on ticks through entomological parameters by comparing the rejection percentage, engorgement percentage, percentage of UTE ticks and engorgement weight between treatment and control group of ticks (LUC injected tick group).

3.9.2 Evaluation by Quantification of Targeted Genes

1. For relative quantification of targeted genes in silenced *H. anatolicum* ticks, design primers with modified parameters like melting temperature (T_m) between 55 and 60 °C, primer length between 17 and 25 mer and amplicon length between 80 and 200 bp to get best primer pair with zero or negative penalty using Primer Express 3.0.1 software (Applied Biosystem, USA) (*see* **Note 5**). Primers used for the qPCR studies, are detailed in the Table 1.

2. Collect the different life stages of tick sample during normal tick rearing process like eggs, larvae, engorged larvae, unfed nymphs, engorged nymphs, unfed adult males, unfed adult females, frustrated females (tick released on animal but not allowed them to feed for 24 h), partially fed females, engorged females and fed males. Clean the different stages of ticks collected from animals in sequential manner with excess tap water, 3% H_2O_2, distilled water, 70% ethanol and finally with distilled water. Dry the cleaned samples by wiping with paper towel. Weigh each stage of tick to 50 mg and make six replicates, store in 1.5 ml microcentrifuge tube at −80 °C for RNA isolation.

3. Extract the total RNA from the 50 mg of each sample using Trizol® reagent as described earlier. Check the quality and quantity of RNA, aliquot and store at −80 °C. Carry out the cDNA synthesis by reverse transcription of total RNA using iScript™ cDNA synthesis Kit (Bio-Rad, USA) according to the manufacturer's instructions.

4. Standardize the PCR conditions for each target in Veriti 96 Well Thermal Cycler (Applied Biosystem, USA) using Dream Taq® DNA polymerase. Apply, the standardized conditions on real-time thermal cycler (Step One Plus Real-Time PCR System, Applied Biosystem, USA).

5. Test the qPCR primers first for any nonspecific reactions like self-dimer, cross-dimer and hairpin formation. Prepare a standard 10 μl reaction mixture containing $2\times$ Fast SYBR® green master mix, forward and reverse primer (10 mM each), and finally add cDNA (10 ng/μl) in triplicate wells and keep another three wells as non-template control. Close the strips, spin and load in real-time thermal cycler with following cyclical conditions:

Initial denaturation	95 °C	20 s	
Denaturation	95 °C	3 s	40 cycles
Annealing/extension	60 °C	30 s	

Melt curve: 60–95 °C (0.3 °C increment), 15 s per step.

If the reaction shows non-specific amplification in NTC (C_T value below 34), then further standardize the reaction by modifying the primer concentration by designing the "Primer matrix" (*see* **Note 6**).

6. Determine the R^2 value, amplification efficiency (*E*%) of the primers and construct the linear standard curve by plotting the log copy number against cycle threshold value (C_T) obtained during amplification at each dilution of the sample.

7. Dilute the cDNA sample to tenfold with initial concentration of 50 ng to a final concentration of 0.005 ng. Set a PCR in 10 μl volume in triplicate using 2 μl of cDNA, 5 μl of SYBR® green master mix, standard primer quantity (according to the results of primer matrix) and make final volume of 10 μl with NFW.

8. Set the qPCR reaction in StepOnePlus Real-Time PCR System base programme "Quantitation-Standard curve" which automatically plot the standard curve and give the value of slope, *Y*-intercept, R^2 and *E*%. Alternatively, enter the raw data of C_T value against log concentration of cDNA in MS-Excel sheet and obtain the linear regression curve to determine the slope value (*m*) and square of correlation co-efficient (R^2). Determine the efficiency of the qPCR from the formulae:

$$E = 10^{-1/\text{slope}}$$

9. For analysis of relative expression of gene of interest, select the set of housekeeping genes like elongation factor 1-alpha (EF1-alpha) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control.

10. Prepare the cDNA from 1 μg of total RNA isolated from each life stage and use it in 10 μl volume in triplicate reactions. Prepare the reaction mixture by mixing 2 μl cDNA, 5 μl SYBR$^®$ green master mix, 0.1 μl each of forward and reverse primer and make up the volume with NFW up to 10 μl, spin the MicroAmp$^®$ Fast Optical 96 well reaction plate covered with Optical Adhesive cover (Applied Biosystem), at $385 \times g$ for 2 min and load in StepOnePlus real-time PCR system. Set the PCR reaction in PCR program "Quantitation–Comparative C_T ($\Delta\Delta C_T$)" with following cycling conditions:

Initial denaturation	95 °C	20 s	
Denaturation	95 °C	3 s	40 cycles
Annealing/extension	60 °C	30 s	

Melt curve: 60–95 °C (0.3 °C increment), 15 s per step.

Use at least three biological replicates in a triplicate reaction (technical replicates) for the quantification of each targeted gene from individual sample prepared from each stage.

11. For relative quantification of gene expression follow the method described by Livak and Schmittgen [17]. Use geometric mean of C_T value of two housekeeping genes viz. GAPDH and EF 1-alpha for the normalization of gene expression. Considering the 100% (±5%) efficiency of qPCR primer, use $2^{-\Delta\Delta CT}$ method to calculate the fold change of gene expression in test sample compared to control/reference sample. Employ One-way ANOVA and the Tukey test to compare the fold change of gene expression of treatment group against control at $p < 0.05$ of significance.

$$\%\text{Efficiency} = (E - 1) \times 100$$

12. Quantify the expression of silenced genes in fully engorged ticks, ticks unable to engorge (UTE) and in partially fed adult female ticks collected after 72 h and 96 h of release on calf. Evaluate the effect of RNAi on tick by comparing the fold change in gene expression in dsRNA injected ticks compared with control ticks.

3.9.3 Evaluation of Impact of RNAi on Cellular Architecture of Oocyte of Injected Ticks

Tick Dissection and Fixation of Sample

1. Initially, anesthetize the *H. anatolicum* ticks by giving thermal shock (keeping at 4 °C for 10 min).

2. Fill the melted paraffin in circular stainless-steel lid of 10 cm diameter, height of 10 mm and allow to solidify at room temperature.

3. Fix the anesthetized ticks with the help of hot soldering iron rod in dissection plate.

4. Make a transverse nick on the anterior end of the cuticle between basis capitulum and anterior end of scutum of tick.

Then extend the nick to the posterior median point through the right lateral border of the cuticular body.

5. Cut the right side of the cuticle by rotating the stainless-steel lid anti clock wise with left hand. After cutting right side, same procedure is applied to left side.

6. After cutting both the sides, lift the dorsal cuticle gently from the anterior end and detach the attachments from visceral organs with a teasing needle.

7. Spray chilled PBS on the viscera to remove blood and gut to give a clear view of the ovary. Then gently lift the whole ovary and transfer to chilled PBS for washing and then immediately fix with 4% paraformaldehyde for 24 h for Hematoxylin and Eosin (H&E) staining.

Dehydration

Cut the ovarian tissues into small pieces of 4 mm size and then dehydrate in increasing ethanol concentrations (70, 80, 90, and 95%) for 15 min each in a cavity block at room temperature.

Infiltration and Embedding in Glycol Methacrylate Resin

1. Use JB-4 Embedding Kit (Sigma-Aldrich, USA) for infiltration and embedding of ovarian tissue.

2. Use infiltration media 8–10 times of the volume of the specimen (~1 ml) without exposure to heat or light for proper infiltration.

3. Gently shake the cavity block and change the fluid for three times at 10 min interval.

4. Then transfer the tissues to an Eppendorf tube containing the infiltration solution with a custom-made scoop (4 × 2 mm) and allow the tissues for complete saturation with the infiltration solution (*see* **Note 7**). After 90 min, change the infiltration solution again and keep the tissues for 24 h in the refrigerator at 4 °C.

5. Just prior to the embedding procedure, mix 25 ml of freshly prepared infiltration solution and 1 ml of JB-4 solution B thoroughly and start the embedding process. At a time, prepare only 5 ml embedding solution (*see* **Note 8**). Pour the embedding solution into a polyethylene BEEM® embedding (Size 3) capsules (Polysciences, Inc., USA) and immediately transfer the tissues into the solution and orient the tissue with a teasing needle. Close the cap of the embedding capsule and transfer to vacuum desiccators fixed at not more than 15 psi, filled with ice to provide a temperature of 2–8 °C required for exothermic reaction during the resin polymerization. After overnight polymerization under anaerobic condition, remove the non-polymerized syrup appeared on the top of the block by inverting the tube and allow it to dry in desiccators.

Microtomy

1. Remove the excess embedding material from the embedding capsule by longitudinally cutting on one side of the capsule and trim the excess resin.

2. Place the trimmed block on the feeding arm of ultramicrotome (Leica EM UC7) with the chuck holder.

3. Arrange the glass knife made out of glass knife strips with the help of glass knife maker (Leica EM KMR3) at the correct angle to cut the section evenly at 3 μm thickness. Rotate the microtome manually to produce a single section on the glass knife.

4. Gently pick the corner of the section with a fine tweezer (Tweezers, 7 Dumont Inox, Biology grade, Poly Sciences, Inc-USA) and release the section one at a time on each droplets of water already placed on the glass slide, without touching the forceps on water, to unwrinkled and flatten on the water. Dry the sections on the slide on a hot plate setting at 50 °C to evaporate the water quickly and to adhere the section to the slide firmly.

H and E Staining

The staining of the JB4 resin section was done as per the protocol for the zebra fish embryo section in JB 4 resin [18], with minor modification.

1. Dip the slides in Harry'ʰ Hematoxylin for 5 min followed by rinsing under running tap water for 2 min.

2. Destain using acid water for 20 s and once again rinse the slides under running tap water for 1 min.

3. Dip the slides in Scott's tap water substitute for 45 s and rinse with running tap water for 3 min.

4. Afterwards dip the slides in the Eosin dye for 3 min and rinse under running tap water for 5 min.

5. Dry the slides at room temperature or heated on low settings on a hot plate and mount the section with cover slip using DPX as mounting medium.

6. Observe the sections under high resolution microscope attached with photographic attachment and annotation software (Fig. 3).

4   Notes

1. Prevent air bubbles during pouring of boiled gel in gel casting tray.

2. Since, custom designed primers are having 20 bases T7 promoter sequence at 5′ ends, the PCR conditions may be

A. Normal tick oocyte   **B. dsRNA injected tick oocytes showing changes**
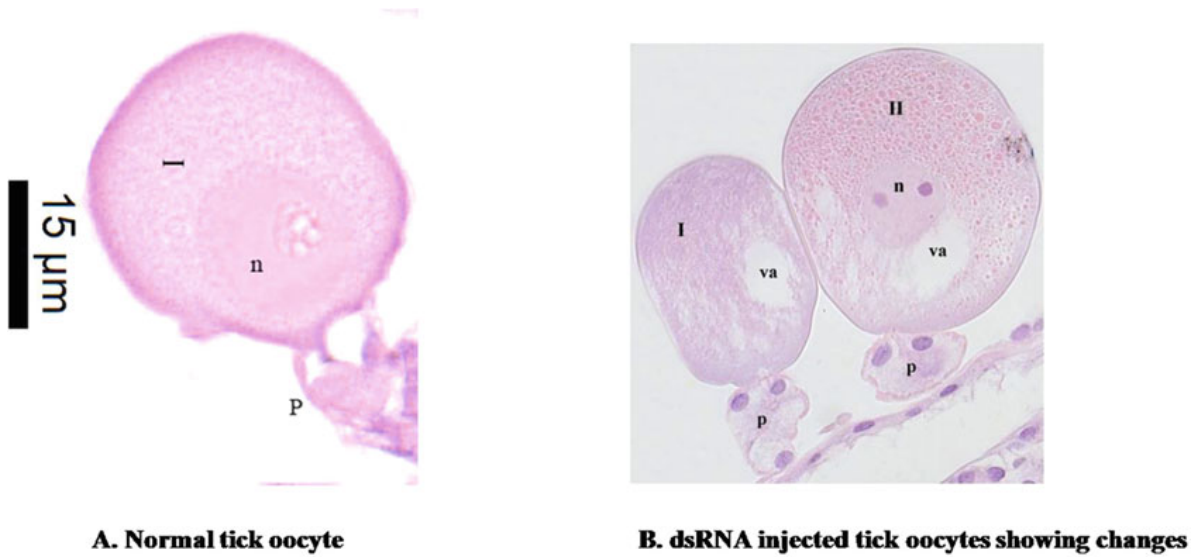
Fig. 3 Histoarchitecture of oocyte of *H. anatolicum* (**a**) normal tick oocyte; (**b**) ds RNA injected tick oocyte (I—type-I oocyte, II—type-II oocyte, n—nucleus, va—vacuole, p—pedicle)

designed in two amplification steps to get amplification of desired product.

3. Anterior half of the ticks were sandwiched between the tapes and postero-ventral side is exposed for injection and it will enable to conduct experiment to conduct easily.

4. Blow the air to activate ticks.

5. Design primers in such a way that one of the primers forward or reverse should be outside the region of gene sequence selected for dsRNA preparation.

6. Real-time quantification of targeted transcript is based on the relationship between the initial template amount and C_T value obtained during amplification, an optimization of assay is very much essential for precise and reproducible quantification of target. For Primer matrix, both forward and reverse primers were kept at different concentration in qPCR reactions, as shown below:

Forward primer quantity (10 pmole/µl)			
Reverse primer quantity (10 pmole/µl)	0.1 µl	0.2 µl	0.3 µl
0.1 µl	0.1/0.1	0.2/0.1	0.3/0.1
0.2 µl	0.1/0.2	0.2/0.2	0.3/0.2
0.3 µl	0.1/0.3	0.2/0.3	0.3/0.3

7. Complete saturation of the tissues will be judged by the level of transparency of the tissue.

8. Always prepare infiltration solution fresh.

5　Concluding Notes

5.1　Future Approaches and Technologies for Novel Anti-Tick Vaccine Design

Discovering the Achilles heel of Ixodid ticks is not an easy task considering the comparatively large genome size with complex life cycle of the Ixodid ticks. Robust and high throughput target screening with cutting-edge genomic technologies combined with appropriate vaccine platforms is the way forward to develop an effective anti-tick vaccine. Recent advances in robust parallel nucleotide sequencing and gene editing technologies offer new hope of finding the most effective target leads for anti-tick vaccine. The emerging vaccine platforms such as glycoconjugate vaccine, microbiome targeting vaccines and viral vectored vaccine loaded with the most effective vaccine targets are expected to make a paradigm shift in anti-tick vaccine technology. The future of anti-tick vaccine technology relies on using cutting-edge technologies for target identification, screening and appropriate vaccine platform/design.

5.1.1　Target Identification and Screening of Anti-Tick Vaccine Candidate Antigens

1. Next-Generation Sequencing (NGS) Technology in target identification: an aid in Reverse genetics approach.

    (a) The huge size and complex genome of Ixodid ticks warrant advanced third generation NGS technology or long read sequencing technology to discern the complete genome with best possible genome coverage. PacBio and Oxford Nanopore NGS technologies are based on long read sequencing approach which can give superior coverage of the tick genome with best possible resolution of the repetitive genomic regions [19].

    (b) Gene copy numbers and alternate splicing: High coverage and superior resolution on repetitive genomic region are important to know the copy number of genes, identify the single copy genes, and understand the orphan genes which lack detectable sequence homology to genes in pre-existing databases [20]. The whole transcriptome with the whole genome sequence assembly will also help us to identify the critical genes involved in alternate splicing. When gene products with alternate isoforms are targeted the selection pressure can easily be overcome by the organisms with other isoforms. Hence, targeting a single copy gene is involved in a critical biological function of the ticks or single copy gene with alternate splicing activity would be the perfect target for an anti-tick vaccine.

(c) Epigenetics: Single Molecule Real-Time Sequencing (SMART) technology used by PacBio third generation NGS technology can identify the epigenetic modifications such as methylation of DNA bases which regulates the gene expression in ticks. DNA methylation at the fifth position of cytosine (5mC) and at the sixth position of adenine (6 mA) were found in all life stages of *Ixodes ricinus* ticks and there are several DNA methyl transferase enzymes thought to be involved in this process [21]. Understanding the epigenetic modification using the NGS technology and the proportion of epigenetic modification of DNA bases in tick species is expected to yield new lead targets such as enzymes involved in the epigenetic modification of DNA bases.

(d) Genetic diversity of ticks: Variation in the efficacy of Bm86 based anti-tick vaccine was attributed to the genetic diversity of the ticks and allelic variation in the Bm86 gene. Considering the large size of Ixodid tick genome, single gene or few genes-based clades typing of Ixodid ticks is not a representative sampling of the large tick genome and not expected to give the appropriate clade typing. Genetic diversity of Ixodid ticks based on whole genome sequences from different geographical regions will expose the existence of different clades or strains and types of ixodid tick species which in turn help us to design region specific anti-tick vaccines.

5.1.2 Anti-Tick Vaccine Target Screening

1. CRISPR/Cas9 technology for *In vivo* screening of anti-tick vaccine targets: The lead anti-tick candidate gene targets can be screened for their phenotype alteration in Ixodid ticks using CRISPR-Cas9 gene editing technology. Unlike RNAi technology, the CRISPR-Cas9 gene editing technology induces permanent and heritable genetic changes in ticks and the phenotypic change can be studied in both homozygous and heterozygous conditions. Successful targeted mutagenesis using CRISPR-Cas9 in the spider mite, *Tetranychus urticae* [22] provided great hope for use of CRISPR-Cas9 gene editing technology in Ixodid ticks to screen the anti-tick vaccine target genes.

2. Endosome encapsulated with Cas9 (RNA guided DNA endonuclease) and gRNA (tracrRNA with crRNA) targeting the candidate gene in the tick genome can be introduced into the tick embryo during early embryonic stage either to knock out the gene or edit the gene with mutations. The gene edited or gene deleted tick larvae can be selected based on fluorescent based markers and propagated to study the phenotypic changes. If the target gene is critical for the survival of the

ticks, no surviving tick larvae would emerge out the mutagenesis experiment. A library of gRNA targeting different genes can be synthesized and a high throughput screening of the target genes is possible.

5.2 Next-Generation Anti-Tick Vaccines Designs

1. Tick microbiome-based vaccines: Ixodid ticks carry diverse group of non-pathogenic commensal and symbiotic microorganisms. The biology of symbionts and their effect on ticks remain largely unexplored. Rickettsia, Francisella, and Coxiella genera are the common symbionts of ixodid ticks [23]. Obligate or primary symbionts of ticks are the microorganisms that support normal tick development and various essential functions; thus, considered as potential vaccine targets. Killed primary symbionts culture or recombinant antigens of symbionts can be used to develop a vaccine against the ticks. Laboratory culture of primary symbionts of ticks or any insects would be a challenging task and needs cutting-edge technology to simulate the micro-habitat of tick organelles in laboratory conditions. However, the recent advances in the next-generation sequencing offers the whole genome sequencing of the primary symbionts of ticks and development of recombinant antigen based anti-tick vaccine using the antigens of primary symbionts is possible.

2. Glycoconjugate anti-tick vaccine: Glycotopes are the epitopes present in the glycan moiety of the organisms which are recognized by the host immune system. The glycotopes of ixodid ticks are not explored for developing an effective anti-tick vaccine. The tick glycoprotein especially from the midgut can be isolated and conjugated to the protein carriers such as tetanus toxoid, diphtheria toxoid, protein D or any suitable tick antigen. Glycans of various size, structure and composition can be synthesized using "Glyconeer" an automated oligosaccharide synthesizer which uses automated glycan assembly on solid phase. The purified glycans can be covalently coupled with carrier protein and resulting glycoconjugates can be used as a vaccine. Alternatively, Protein Glycan Coupling Technology (PGCT) which facilitates the *in vitro* transfer of glycans to a recombinant acceptor protein using the glycosylating enzyme can be used in large-scale synthesis of the glycoconjugates. The antigen presenting cells present the oligosaccharides along with carrier protein to T-cells to elicit adaptive immune response against the glycan moiety [24]. Since midgut and salivary gland of ixodid tick is an oligosaccharide or glycoprotein rich environment, glycoconjugate vaccine platform against ticks is a more attractive approach and promising one.

3. Viral vectored anti-tick vaccines: Viral vectored vaccine for Ixodid ticks is an unexplored option. A non-replicative

adenovirus such as Ad5 expressing tick antigens is expected to elicit strong cytotoxic T-cell and humoral immune response against the tick antigens. Priming with viral vectored anti-tick vaccine and boosted with recombinant antigen, would give strong and long duration immunity against ticks. The advantage of viral vectored vaccine is, more than one tick antigen can be co-expressed to give better immunity for extended duration.

4. Old is gold—Native midgut antigen: Large-scale production of native midgut antigens to meet the commercial scale is the bottleneck in native antigen based anti-tick vaccine technology. Creating an immortal Ixodid tick midgut cell line would eliminate the bottleneck in the large-scale production of native antigens and thought to be a promising approach. Creating a midgut germ cell based immortal cell line or chemically immortalized midgut cell line is the way forward to make the native midgut antigen based anti-tick vaccine commercially successful.

Acknowledgments

Conflict of Interest: There is no conflict of interest from authors.

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Chapter 19

Host Immunization with Recombinant Tick Antigen and Evaluation of Host Immune Response

Kodai Kusakisako, Takeshi Miyata, Kozo Fujisaki, and Tetsuya Tanaka

Abstract

Ticks are classified as hematophagous arthropods and transfer a variety of pathogens—such as viruses, bacteria, and protozoans—to vertebrate hosts during blood feeding. These transmitted pathogens cause infectious diseases that continue to affect both humans and animals worldwide. Chemical acaricides are commonly used for tick control to prevent infectious diseases. However, the continuous use of acaricides leads to the emergence of acaricide-resistant tick species; thus, alternative methods for tick control are necessary. Vaccination of vertebrate hosts with tick-derived molecules is considered to be a better alternative against ticks than chemical acaricides because ticks feed on host blood for several days and also concentrate the host blood with antibodies. On the other hand, the host's immune responses against pathogens mainly take two pathways—Th1 (cell-mediated immunity) and Th2 (humoral immunity) pathways. Thus, the vaccine can suggest which immune pathway is more important for vaccination. This chapter describes the procedures of immunizing laboratory animals—mice—with a recombinant tick protein for the preliminary evaluation of its potential as an anti-tick vaccine candidate. In addition, the method of evaluating the antigen-specific antibody production in the host using ELISA is described, as is the subsequent tick-infestation challenge for determining the effectiveness of vaccination.

Key words Tick, Tick-derived antigen, Vaccination, Host immune response, Recombinant protein purification for vaccination, Tick challenge to the model animal

1 Introduction

Ticks are classified as hematophagous arthropods; during blood feeding, they transfer to vertebrate hosts a variety of pathogens such as viruses [e.g., tick-borne encephalitis (TBE) virus and severe fever with thrombocytopenia syndrome (SFTS) virus], bacteria (e.g., *B. burgdorferi* and spotted fever rickettsia), and protozoans (e.g., *Babesia* and *Theileria* parasites) [1, 2]. The infectious diseases caused by these pathogens, called tick-borne diseases (TBDs), continue to affect both humans and animals worldwide [3]. Thus, researchers have made efforts to regulate TBDs by controlling

ticks using chemical acaricides. However, the continuous use of acaricides leads to the emergence of acaricide-resistant tick species. Therefore, alternative methods of tick control are necessary.

The vaccination of vertebrate hosts with tick-derived molecules is considered better than using chemical acaricides against ticks [4] because ticks feed on the host blood for several days and also concentrate the host blood with antibodies; this concentration of the blood leads to high titers of antibodies in ticks. On the other hand, the host immune responses against antigens derived from parasites mainly have two pathways, Th1 and Th2. Normally, the Th1 immune pathway is related to cell-mediated immunity, while the Th2 immune pathway is associated with humoral immunity. In animal models challenged with pathogens such as *Schistosoma mansoni* [5], vaccines inducing Th1 immune responses have been proven highly effective at preventing infections, whereas vaccines inducing Th2 immune responses have suppressed the cell-mediated inflammation related to infection, which is considered to increase the host's susceptibility to infection by pathogens. Therefore, although Th1 immune responses are a key to protecting against most infections, vaccines and passive immunization rely on Th2 immune responses [6].

This chapter describes the procedures for immunizing laboratory animals, mice, with a recombinant tick protein for the preliminary evaluation of its potential as an anti-tick vaccine candidate. In our laboratory, we use an *E. coli*-based expression system, the most widely used system for the synthesis of recombinant proteins [7], which will be described here in more detail. We also describe how to evaluate antigen-specific IgG antibodies production—such as total IgG, IgG1, and IgG2a—in the host using enzyme-linked immunosorbent assay (ELISA) to determine whether the antigen will activate the Th1 or Th2 immune pathway. In addition, we describe the subsequent tick-infestation challenge for determining the effectiveness of the vaccination.

2   Materials

2.1   For Recombinant Protein Preparation

1. Luria-Bertani (LB) broth: Dissolve 25 g of LB powder for every 1 L water. Autoclave at 121 °C for 15 min, and then cool at room temperature before storage or use. Add ampicillin at a final concentration of 50 μg/ml.

2. *E. coli* stock expressing recombinant protein: Place transformed competent *E. coli* BL21 (DE3) cells, containing the plasmid (e.g., pRSET C; Invitrogen, Carlsbad, CA, USA) inserted with the gene encoding the target protein, in a cryotube with equal volumes (1 ml) of 30% glycerol (final concentration is 15%) and LB broth with ampicillin. Store at −80 °C.

3. One M isopropyl β-D-thiogalactopyranoside (IPTG): Prepare 1 ml aliquots, and store at −20 °C until use.

4. Phosphate-buffered saline (PBS; 10×): Mix a solution of 1.37 M NaCl, 100 mM Na_2HPO_4, 27 mM KCl, 18 mM KH_2PO_4. Autoclave at 121 °C for 15 min, and then store at room temperature.

5. Urea Solution: Prepare a solution of 6 M urea, 20 mM Tris–HCl (pH 7.5), 500 mM NaCl.

2.2 For Recombinant Protein Purification by Fast Protein Liquid Chromatography (FPLC)

1. Nickel sepharose column for Histidine (His)-binding: HisTrap FF (GE Healthcare, Uppsala, Sweden) 1 ml. Store at 4 °C.

2. Binding buffer: 20 mM Tris–HCl (pH 7.4), 500 mM NaCl. This solution should be filtered using a 0.45 μm syringe filter before use.

3. Elution buffer: 20 mM Tris–HCl (pH 7.4), 500 mM NaCl, 500 mM imidazole. As with the binding buffer, this also should be filtered before use.

4. BioLogic DuoFlow chromatography system (Bio-Rad, USA).

2.3 Mouse Immunization

1. Mice: Female 4-week-old BALB/c mice. They should not have been infested before with ticks or used in any other experiments.

2. Three types of mixed anesthetic agents: 0.75 mg/kg medetomidine, 4 mg/kg midazolam, 5 mg/kg butorphanol. The anesthetic agent is administrated intraperitoneally at a concentration of 0.1 ml/10 g mouse body weight.

3. Freund's incomplete adjuvant: Store at 4 °C until use.

4. 2 ml glass syringes and micro-emulsifying needle: sterilized by autoclaving at 121 °C for 15 min.

5. 28 G hypodermic needles: Single use, disposable.

2.4 Measurement of Antibody Titers by ELISA

1. For blood collection: Collect blood from the orbital sinus under anesthesia.

2. Mouse serum as a primary antibody: After collecting blood, let it stand at room temperature for at least 30 min. Centrifuge at $22,140 \times g$ at 4 °C for 10 min. Transfer the serum to a new tube, and store at −20 °C until use.

3. ELISA plate.

4. Recombinant protein: Store at −20 °C and thaw prior to use.

5. Carbonate buffer: 35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6. Store at 4 °C.

6. 1× PBS with 0.05% Tween 20 (PBS-T).

7. Blocking solution: 1% skimmed milk dissolved in PBS-T.

8. Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin: Use as a secondary antibody for the evaluation of total IgG titers. Store at 4 °C.

9. HRP-conjugated goat anti-mouse IgG1: Use as a secondary antibody for the evaluation of IgG1 titers as a Th1 immune pathway marker. Store at 4 °C.

10. HRP-conjugated goat anti-mouse IgG2a: Use as a secondary antibody for the evaluation of IgG1 titers as a Th2 immune pathway marker. Store at 4 °C.

11. SureBlue™ TMB microwell peroxidase substrate: Store at 4 °C.

12. 0.6 N H_2SO_4: Store at room temperature.

13. 1 N HCl: Store at room temperature.

14. Microplate reader with 450 nm filter.

2.5 Tick-Infestation Challenge

1. Electric shaver.

2. Feeding units: 15 ml Falcon tube, sewn cloth, elastic tape, insulating tape, glue, 23 G needle (this unit technique originated in Anisuzzaman et al. [8]).

3. Containers for storing engorged ticks.

3 Methods

3.1 Large-Scale Recombinant Protein Synthesis and Extraction

1. Prepare a pre-culture by inoculating *E. coli* stock in 10 ml LB broth with ampicillin. Incubate at 37 °C with shaking at 144 rpm using a multi shaker (MMS-3020, EYELA, Tokyo, Japan) overnight or until the absorbance at OD_{600} is around 1–2.

2. Add the pre-culture to 500 ml LB broth with ampicillin, and incubate at 37 °C with shaking at 170 rpm until $OD_{600} = 0.4$–0.6.

3. Add IPTG at a final concentration of 1 mM, and incubate further at 37 °C with shaking at 103 rpm using a rotary shaker for 6 h or overnight.

4. Collect the cells by centrifugation at 3,350 × *g* for 30 min at 4 °C. Remove the supernatant (medium), and resuspend the cells in 5 ml diluted PBS.

5. Transfer the cell suspension to a 50 ml tube, and place on ice.

6. Set an ultrasonic processor as follows: amplitude—30, timer— 1 min, pulser—1. Sonicate the cell suspension three times while on ice.

7. Centrifuge at 3,350 × *g* for 5 min at 4 °C. Repeat sonication as described above, and centrifuge again.

8. After another round of sonication, centrifuge at 3,350 × g for 30 min at 4 °C.

9. Transfer the supernatant to a new tube. This is the PBS-soluble fraction. Resuspend the pellet in 10 ml sterilized high-purity distilled water.

10. Sonicate and centrifuge as in **steps 6–8**.

11. After the third centrifugation, transfer the supernatant to a new tube. This is the water-soluble fraction. Add 10 ml of 6 M urea solution to the pellet without disturbing it.

12. Place the tube containing the pellet and urea solution in an automatic rotator overnight at 4 °C, and set it at a very low speed. The pellet should be completely dissolved.

13. Centrifuge at 3,350 × g for 30 min at 4 °C until the next day.

14. Obtain the supernatant, and transfer it into a new tube. This is the urea-soluble fraction. Resuspend the remaining pellet (insoluble fraction) in 10 ml urea as the insoluble fraction (*see* **Note 1**).

3.2 Purification of Recombinant Protein

1. Confirm the presence of protein through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2. Clean the tubes of the BioLogic DuoFlow chromatography machine with high-purity distilled water, and then put binding buffer in one tube and elution buffer in another.

3. Wash the tubes with the respective buffers by running the machine for 5 ml for each buffer.

4. Attach the HisTrap FF column to the machine. Wash the column with elution buffer first for 5 ml, followed by binding buffer for 5 ml, and finally by high-purity distilled water for 10 ml.

5. Prepare a running protocol (*see* **Note 2**). A sample protocol is shown in Table 1.

6. Program the machine to collect fractions at a volume of 1 ml per tube while running the elution buffer.

7. Run the programmed protocol. After running, wash the column with 5 ml of the following in order: elution buffer, high-purity water, 1 M NaOH (flow rate 0.4 ml/min), high-purity distilled water, binding buffer, 20% ethanol.

8. Check the recombinant protein content of the fractions by SDS-PAGE.

9. Pool the fractions with the recombinant protein in a dialysis bag.

10. Place the dialysis bag in a beaker with a magnetic stirrer containing 1 L of PBS solution. Place the dialysis setup at 4 °C. Set

Table 1
A sample protocol for gradient elution

Loading steps (order of loading buffers)	Running volume (ml)
1. Binding buffer	5
2. PBS or water-soluble fraction (sample)	20
3. Binding buffer	30
4. Gradient (*see* **Note 3**): Binding buffer/Elution buffer	30→0/0→30
5. Elution buffer	5
6. Binding buffer	10

Step 1. is a phase of column equilibration. Step 2. is a phase of recombinant proteins binding to column. Step 3. is a washing phase of the column. Step 4. is the gradient elution phase of the recombinant proteins and Step 5. is a final elution phase of the proteins. Step 6. is the washing phase of column

the speed of the stirrer to the lowest setting, and keep it overnight.

11. The next day, transfer the dialysis bag to another beaker containing 1 L PBS, and keep it in a similar condition as above overnight.

12. Check the protein using SDS-PAGE. Also check the concentration before storing in small aliquots at −20 °C.

3.3 Mouse Immunization

1. Prepare the vaccine: Draw equal amounts of adjuvant and recombinant protein in two separate glass syringes, and connect the syringes with a micro-emulsifying needle. To mix manually, alternately push the syringe plunger toward the opposite syringe to transfer its contents. Do this slowly and repeatedly for at least 10 min to ensure that the vaccine has been mixed thoroughly. Finally, place all of the vaccine mixture in one syringe, and replace the emulsifying needle with a hypodermic needle. For the control mouse, prepare a syringe containing 100 μl of adjuvant with PBS.

2. Take the mouse out of the cage by grasping the tail. Shave the hair on the back of the mouse using the electric shaver.

3. To do a subcutaneous injection, hold the mouse using a simplified holder to apply tension to the mouse skin (Fig. 1). Disinfect the injection site using cotton soaked in 70% ethanol. Insert the needle parallel to the mouse's back, making sure that the needle does not come out of the skin. Aspirate to ensure the proper placement of the needle, and then slowly inject all of the vaccine mixture while pulling out slowly.

4. Repeat the vaccination at two-week intervals to increase the antibody titer on the other side of first inoculation (Fig. 2).

Fig. 1 A simplified holder for mouse. The holder consists of a chopping board and a clothing pin. The mouse is hold around the neck and its tail is pulled to apply a tension on its back

3.4 Blood Collection and Measurement of Antibody Titer

1. Collect blood from the orbital sinus of mice under anesthesia.

2. Disinfect the collection site using cotton soaked in 70% ethanol.

3. Prepare the serum as described in Subheading 2.4.

4. To coat an ELISA plate with recombinant protein, dilute the recombinant protein in carbonate buffer in a tube to a concentration of 1 μg/ml. Put 100 μl of the recombinant protein solution in each well using a multichannel pipette. Cover the plate, and incubate overnight at 4 °C.

5. The next day, discard the recombinant protein solution, and then wash the ELISA plate three times with PBS-T. Tap the plate in a pile of tissue to remove the remaining wash solution after the third wash.

6. Place 150 μl of blocking solution per well, cover the plate, and incubate at room temperature for 1 h.

Fig. 2 The vaccine agent inoculation sites on the mouse. Blue circles indicate the inoculation sites of the vaccine mixture. A side is for first inoculation and the other side is for second inoculation two weeks from the first inoculation

7. Remove the blocking buffer, and then wash the plate four times with PBS-T.

8. Incubate the plate with the primary antibody diluted serially by placing 100 μl of blocking solution in all wells and then adding the serum in the leftmost column; mix several times using the pipette. Next, transfer a certain amount of the diluted antibody solution to the next well on its right, mixing several times before and after transferring. Proceed with the serial dilution until the last column has been reached. Also prepare a row containing only blocking solution to serve as "blank." Cover the plate, and incubate at room temperature for 1 h.

9. Remove the primary antibody solution, and then wash six times with PBS-T. Tap the plate in a pile of tissue to remove the remaining wash solution after the last wash.

10. Place 100 μl of diluted HRP-conjugated secondary antibody in blocking solution (1:4,000 dilution) in each well. Incubate the plate at room temperature for 1 h.

11. Remove the second antibody solution, and then wash as described in **step 9**.

12. Place 100 μl of TMB HRP Microwell peroxidase substrate in each well. Cover the plate with aluminum foil, and then incubate at 37 °C for 30 min.

13. Retrieve the ELISA plate from the incubator, and stop the reaction by adding 100 μl of freshly prepared acid solution, consisting of equal volumes of 0.6 N H_2SO_4 and 1 N HCl per well.

14. Read the absorbance in a microplate reader at 450 nm.

15. Check the antibody titer before immunization and a week after each vaccination.

3.5 Tick-Infestation Challenge

1. Shave the back of the mouse (Fig. 3a).

2. Put the tick-infestation unit on the shaved back of the mouse (Fig. 3b), and wait until the unit is fixed on the back of mouse with glue.

3. Place the ticks in the unit, and close the cap. If using adult ticks, use 1–2 females and 1 male tick. Otherwise, larvae and nymphs may be put in units of less than 50 and 25 ticks, respectively.

4. Monitor the mice, and check tick attachment twice a day until the ticks fully engorge and drop naturally.

5. Count the number of ticks that fully engorge, and measure their individual weight.

6. Place the engorged ticks on plastic tubes or vials with a cotton plug. If using adult ticks, keep the ticks in individual vials. Otherwise, larvae and nymphs may be kept in groups of 40–100, depending on the size of the container. Place the containers with ticks in a glass chamber with a little water underneath for humidity, and keep at 25 °C.

7. Assess the total number of ticks that successfully engorged divided by the number of infested ticks (engorged rate), the total number of nymphal ticks molted to adult ticks divided by the number of successfully engorged ticks (molting rate), and the survival rate after molting from nymphs to adult ticks.

4   Notes

1. If the recombinant proteins were extracted as the urea-soluble or -insoluble fractions in Subheading 3.1, suitable buffers are different from the buffers used in Subheading 3.2. In the case of the purification of urea-soluble or -insoluble fractions, please *see* Chapter 18 (Galay et al., "Host immunization with recombinant proteins to screen antigens for tick control") of Sunil Thomas (ed.), Vaccine Design: Methods and Protocols, Volume 2: Vaccines for Veterinary Diseases, Methods in Molecular
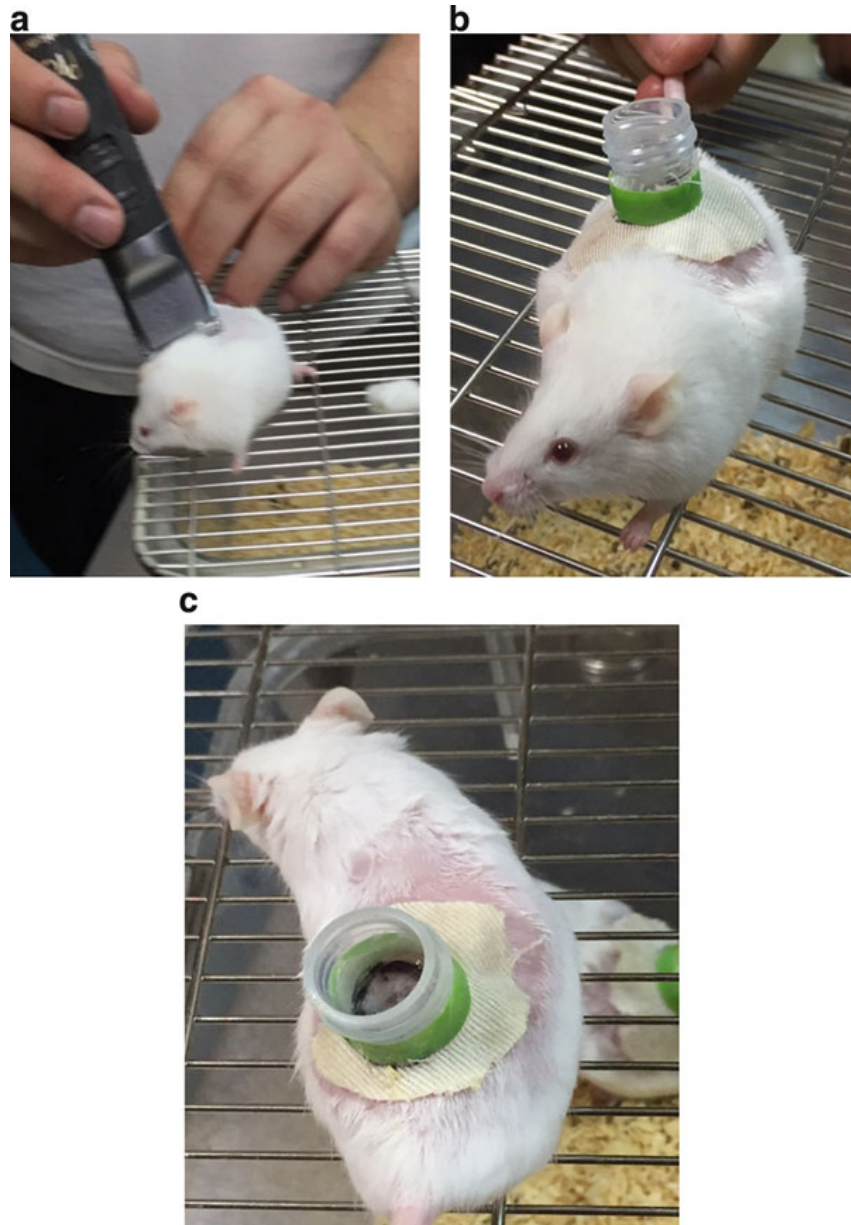
Fig. 3 Mice during the tick challenge. Initially, the back of the mouse is shaved. Then, the tick-infestation unit is put on the shaved back of the mice. Finally, ticks are put in the unit and allowed to feed on the mice. (**a**) The hair on the back is shaved. (**b**) Place the tick-infestation unit on the mouse and fix with glue. (**c**) The tick-infestation unit on the mouse

Biology, vol. 1404, Springer Science+Business Media, New York, 2016.

2. In this chapter, the protocols for purification recombinant protein are for the "PBS-soluble fraction" or the "water-soluble fraction" in Subheading 3.2.

3. This setting involves the gradient method. The loading volumes are from 30 ml to 0 ml (binding buffer) and from

0 ml to 30 ml (elution buffer). In other words, 100% binding buffer is used at the start, and 100% elution buffer is used at the end, indicating that the imidazole concentrations are from 0 mM to 500 mM in the elution step of the recombinant protein. Thus, although dependent on the recombinant protein, this gradient method would be useful for separating *E. coli*-derived proteins from the recombinant proteins because the suitable concentration of imidazole for the elution buffer can be detected.

Acknowledgments

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Chapter 20

Fundamental Tick Vaccinomic Approach to Evade Host Autoimmune Reaction

Seham H. M. Hendawy, Heba F. Alzan, Tetsuya Tanaka, and Mona S. Mahmoud

Abstract

Ticks are obligate hematophagous ectoparasites that infect domestic animals, humans, and wildlife. Ticks can transmit a wide range of pathogens (viruses, rickettsia, bacteria, parasites, etc.), and some of those are of zoonotic importance. Tick-borne diseases have a negative economic impact in several tropical and subtropical countries. With climate change, tick distribution and tick-associated pathogens have increased. Currently, tick control procedures have more environmental drawbacks and there are pitfalls in vaccination process. Since vaccinations have helped to prevent several diseases and infections, several vaccination trials are ongoing to control ticks and tick-borne pathogens. However, autoimmune reactions to vaccinations are reported as an adverse reaction since vaccines were used to protect against disease in humans and animals. The antibodies against the vaccine antigen might harm similar antigen in the host. Therefore, in this chapter, we attempt to shed light on the importance of raising awareness of possible adverse events associated with vaccinations and the methods that should be used to address this problem. In silico and lab work should be performed ahead of the vaccination process to evaluate the vaccine candidates and avoid the vaccination opposing consequences.

Key words Tick control, Tick vaccine, Shared antigen, Recombinant proteins, Autoimmune reaction

1 Introduction

Ticks are considered the second most important obligate hematophagous ectoparasites, next to mosquitoes. They transmit most of the hemoparasitic pathogens (*Babesia*, *Anaplasma*, *Borrelia*, *Theileria*, etc.) that affect human and animal health and welfare [1–3]. Ticks and tick-borne diseases economically impair livestock production and industry through consecutive annual losses in tropical and subtropical areas all over the world. Global environmental

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Seham H. M. Hendawy and Heba F. Alzan contributed equally to this work.

and climatic changes have affected the epidemiology of ticks and tick-borne pathogens by increasing the global spread of ticks and tick-related pathogens affecting both humans and domestic animals [4, 5].

Tick control is dependent mainly on the application of chemical acaricides, the long-term use of which leads to the emergence of acaricide-resistant tick strains, and which contaminate the environment, leading to enzootic instability and the contamination of meat and dairy products. Therefore, the current strategies rely on an unconventional, non-environmentally friendly method to control tick infestations [6, 7]. Since the 1990s, vaccines have provided an ecologically safe and effective alternative to tick control. However, the knowledge gap in understanding the tick's physiological functions in conjunction with the tick–pathogen–host interface represents an obstacle to identifying novel effective antigens for commercial development of new anti-tick vaccines [2, 8].

One of the most critical constraints affecting the development of an anti-tick vaccine candidate is that ticks and their hosts belong to the domain Eukaryota. The selection of vaccine candidates usually depends on antigens, which play vital roles in the tick life cycle that involve in critical biological functions. Those protein antigens can be, at the same time, homologous to their host antigens inducing the host cross immune responses, upon vaccination, which lead to generating an autoimmune reaction. The developed adverse effect is probably created by molecular mimicry mechanism. When the vaccine antigens immunologically similar to the host antigens, that trigger a different immune response when presented to T-cells result in an unusual immune reaction inside the vaccinated host. This unusual immune reaction inside the host body will be against the host normal tissues within different organs generating an inflammatory reaction, autoantibodies, fatigue, pain, impaired mobility, and other non-specific clinical signs on the immunized host [9–13]. Unfortunately, numerous groups worldwide who have performed vaccination trials using full recombinant proteins, synthetic peptides, or chimera, did not detect possible host autoantibodies that could be generated against the host antigen after vaccination [4, 7–9, 14–16]. Although the occurrence of a fatal autoimmune reaction post-vaccination has been less frequently recorded, it could be one of the main obstacles in the registration of effective vaccines for the control of ticks and tick-borne pathogens. To address the problem of the occurrence of autoimmune reaction it should be factored during vaccine design and how it could affect vaccine efficacy and efficiency [17, 18]. It should be noted that the autoimmune reaction phenomenon can vary depending on animal race, age, gender, location, and many other factors, which should be taken into consideration in dealing with this problem.

The diagnosis of autoimmune disorders (autoantibodies), that could occur as a post-vaccine reaction in animals, should be applied in the vaccinated animal. Veterinary autoimmune diagnostic tests are virtually identical to human ones but are modified for their specific species reagents [19–24]. One of the tools used to detect the autoimmune reaction is antinuclear antibodies (ANAs), which are considered a sensitive gold marker for detection of autoimmune disease and represent a specific heterogeneous class of autoantibodies (categorized into autoantibodies to DNA and histones and autoantibodies to extractable nuclear antigens) that are capable of binding and destroying certain structures within the nucleus of the cells [23, 25]. The standard method for ANA detection in the sera of both humans and animals is the indirect immunofluorescent (IIF) technique based on the use of tumor cell lines (the laryngeal carcinoma cell line HEp-2cells [ATCC-CCL 23]) which is now the preferred cell substrate for IIF ANA screening that allows the detection of autoantibodies to several cellular domains, such as the cytoplasm and mitotic apparatus [22, 24].

In the omics era, an amalgamation of in silico bioinformatics analysis, functional genomics, proteomics, transcriptomics, and gene-editing methodologies are fundamental tools in accelerating research and discovery to shape a better future by developing an efficient anti-tick vaccine. These approaches could resolve the complexity of the tick's life cycle and solve the host–pathogen–tick interaction mystery [16, 18, 26].

In the current study, we hypothesize that use of *in silico* bioinformatic analysis within a reverse vaccinomic approach serves as a beneficial tool in order to define the unique epitope in tick proteins to avoid host autoimmunity upon vaccination. In addition, we suggest that tick vaccine candidates should be compared with their host antigens to avoid the immune cross-reaction upon vaccination (Fig. 1), for increasing the vaccine efficiency and efficacy.

2   Materials

2.1   In Silico Data Acquisition for Identifying a Unique Tick-Specific Peptide (Fig. 2)

2.1.1   In Silico Analysis to Identify the Homology Relationship Between the Tick Peptide(s) and its Host(s)

1. The Basic Local Alignment Search Tool (BLAST) for nucleotide (n) sequence (BLASTn).

2. Expasy translate tool.

3. BLASTp tool using a BLOSUM62 matrix.

4. MUSCLE software.

5. MEGA software version X.

6. R studio using Sequir, ggplot2, and reshape2 packages.

7. Multiple Expression motif for Motif Elicitation (MEME) tool version 5.0.5 and PyMol version 2.4.
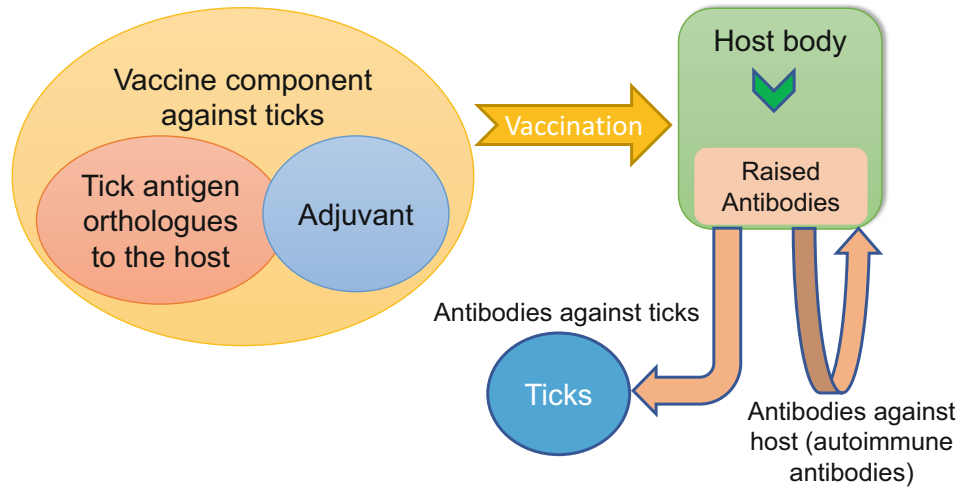
Fig. 1 Schematic representation of the hypothetical mechanism for using orthologues tick antigen differ from their host (s) during the vaccination process
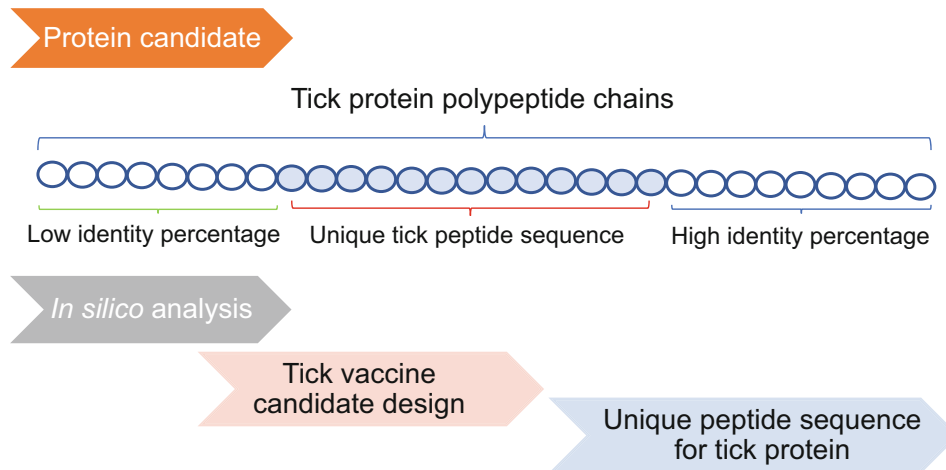
Fig. 2 Schematic representation of the methodology that should be followed in anti-tick vaccine design. Along the tick protein for example, there are areas with high, medium, and low sequence identity between the tick and the host protein as well as a unique peptide sequence for tick. *In silico* analysis should be used as a guide to choose the unique peptide component in the anti-tick vaccine

8. MODELLER version 9.22, (z-DOPE) score, (ProSA-web), and RAMPAGE programs.

2.1.2 In Silico B-Cell Epitopes Prediction

1. Immune Epitope Database Analysis Resource (IEDB) (https://www.iedb.org/).

2.2 Recombinant Protein Production of Tick and Host Antigens

2.2.1 RNA Extraction and RT-PCR

1. Tissue specimen of animal host and tissue dissection of tick vector for RNA extraction.
2. Phosphate-buffered saline (PBS 10×, pH 7.2) is prepared by mixing 1.23 M NaCl, 0.27 M Na_2HPO_4, 0.026 M KH_2PO_4, and 0.027 M KCl. Add a volume of water to 1000 mL. Autoclave at 121 °C for 20 min, keep at room temperature, and prepare 1× upon usage.
3. RNAprotect Tissue Reagent.
4. RNeasy Mini Kit.
5. RNase-Free DNase Set.
6. OneStep RT-PCR Kit.
7. HotStarTaq Master Mix Kit.
8. Nuclease-Free Water.
9. Primers.

2.2.2 Cloning and Expression of Both Animal Host and Tick sp. Recombinant Proteins

1. TOPO® TA vector (Invitrogen, USA).
2. pBAD expression vector.
3. Low-salt Luria–Bertani agar (LBA) plates (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, 1.5% agar).
4. QIAprep Spin Miniprep Kit (Qiagen, Germany).
5. AmpliTaq DNA polymerase (5 U/μL stock).
6. TaqStart antibody (1.1μg/μL stock; Clontech).
7. Incubator.
8. Shaking incubator.
9. Vortex.
10. Thermal cycler.
11. Laminar flow.
12. Cooling centrifuge.

2.2.3 Purification of Both Expressed Recombinant Proteins

1. X-histidine tag supplied by the vector sequence.
2. ProBond Purification System (Life Technologies).
3. ProBond™ nickel-chelating resin.
4. Amicon Ultra-15 centrifugation units.

2.3 Synthetic Peptide Production

1. Regions of the targeted tick protein containing *in silico* predicted B-cell epitope.
2. Keyhole Limpet Hemocyanin (KLH).

1. Specific animal host (cattle, sheep, dogs, etc.).
2. The animal host and tick vector prepared recombinant proteins.

2.4 Host Immunization Using the Validated Animal Host and Tick Recombinant Proteins and the Tick Synthetic Peptide

3. The B-cell epitope synthetic tick peptide.

4. Other supplements and materials as previously described by Rodríguez-Mallon [27] and Galay et al. [28] for developing an anti-tick vaccine and host immunization with recombinant proteins to screen antigens for tick control, respectively.

2.5 Detection of Autoimmune Reaction

2.5.1 In Vitro Autoimmune Analysis

1. Animal host cell line culture (ATCC services).

2. Dulbecco's Modified Eagle Medium (DMEM).

3. Media Supplements: Glucose, L-Glutamine, and Bovine fetal serum.

4. CO_2 incubator.

5. PBS $1\times$ (mentioned in the above section).

6. The cell extract buffer is prepared by mixing 5 mM Tris–HCl buffer, pH 7.4, with 20 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM Iodoacetamide, 2 g/mL Leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF).

7. The tick cell extract is prepared from a tick specimen under study utilizing the previously described cell extract buffer.

8. Non-pyrogenic filters (0.2μm).

9. Cooling centrifuge.

10. Homogenizer.

11. Polyclonal sera are developed against the specific tick peptide and the host and tick full recombinant protein during the previously described immunization experiment. The sera are used at different dilutions (1:100, 1:1000, 1:10000).

12. Secondary antibodies, an anti-IgG species-specific polyclonal antibody conjugated to peroxidase.

13. SDS-PAGE and western blotting buffers are mentioned in the above section.

2.5.2 In Vivo Autoimmune Analysis

1. ANA [antinuclear antibody] HEp-2 IFA Kit (commercially available if the animal host is a dog).

2. HEp-2 cell line and other supplements according to materials published by Dellavance and Andrade [24].

3. Sera collected from the examined animal host.

2.6 Vaccine Efficacy by Tick Challenge

1. The type of tick colony that will be used for the animal challenge will be according to the purpose of the investigation.

2. The material and supplements needed for the animal challenge are mentioned by Rodríguez-Mallon [27] and Galay et al. [28]

for developing an anti-tick vaccine and host immunization with recombinant proteins to screen antigens for tick control, respectively.

3 Methods

3.1 In Silico Data Acquisition to Identify a Unique Tick-Specific Peptide Sequence

3.1.1 In Silico Analysis to Identify the Homology Relationship Between the Tick Peptide(s) and its Host(s) Sequences

1. Analyze the tick gene sequences against the non-redundant nucleotides and their similar host gene(s) deposited in Gen-Bank using BLASTn tool on the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) [29, 30] (*see* **Note 1**).

2. Translate the tick and host gene sequences into open reading frames (ORFs) [31] using the Expasy translate tool (https://www.expasy.org/) at the bioinformatics resource portal of the Swiss Institute of Bioinformatics (SIB) (https://www.sib.swiss/) (*see* **Note 2**).

3. Identify distant evolutionary relationships among the tick and host protein sequences using the Blocks Substitution Matrix (BLOSUM) 62 for BLASTp against the protein database (PDB) at NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) [32–34].

4. Compare the multiple sequence alignment (MSA) of the tick and host protein sequences using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program (https://www.ebi.ac.uk/Tools/msa/muscle/) [35, 36] (*see* **Note 4**).

5. Analyze the change in the sequence composition of the tick and host protein sequence data from species and populations across generations, and construct the phylogenetic tree using the maximum-likelihood method with 1000 bootstraps in Molecular Evolutionary Genetics Analysis (MEGA) software version X (https://www.megasoftware.net/) [37, 38] (*see* **Note 5**).

6. Measure a Pearson correlation matrix between tick and host amino acid sequences and their orthologs using R studio with Sequir, ggplot2, and reshape2 packages (https://www.r-project.org/) [39] (*see* **Note 6**).

7. Analyze the tick and host amino acid sequences in FASTA format for motif-based sequence analysis using (MEME) tool Version 5.0.5 (http://meme-suite.org/tools/meme) [40, 41]. Visualize and analyze the structure superimposition of the atomic structures of the tick and host proteins using 3D modeling in PyMol version 2.4 (https://pymol.informer.com/versions/) (*see* **Note 7**).

8. Calculate homotetrameric homology automodel class of a unique tick-specific peptide by using the MODELLER version 9.22 program (https://salilab.org/modeller/9.22/release.html) [42], and select the best models based on the lowest normalized Discrete Optimized Protein Energy (z-DOPE) score [43] (*see* **Note 8**).

9. Evaluate the model structure using Protein Structure Analysis (ProSA-web) (https://prosa.services.came.sbg.ac.at/prosa.php) and the Ramachandran plot using RAMPAGE in order to select the best model [44–47].

3.1.2 In Silico B-Cell Epitope Prediction

1. Predict linear B-cell epitopes of the tick-specific peptide using IEDB methods at the Immune Epitope Database Analysis Resource (https://www.iedb.org/): Chou and Fasman Beta-Turn Prediction, Emini Surface Accessibility Prediction, Karplus and Schulz Flexibility Prediction, Kolaskar and Tongaonkar Antigenicity, Parker Hydrophilicity Prediction, Bepipred-1.0 Linear Epitope Prediction, and Sequential B-Cell Epitope Predictor [48–53] (*see* **Note 9**).

2. Additionally, conduct a discontinuous B-cell epitope prediction using the DiscoTope method at the Immune Epitope Database Analysis Resource (https://www.iedb.org/) or at (http://www.cbs.dtu.dk/services/DiscoTope/) [54, 55] (*see* **Note 10**).

3.2 Recombinant Protein Production of Tick and Host Antigens

3.2.1 RNA Extraction and RT-PCR

1. Dissect the tick tissues with PBS, pH 7.2, then keep the dissected tick and animal host specimen in RNA protect Tissue Reagent at −80 °C until use (*see* **Note 11**).

2. Extract RNA from the tick and the animal host tissues using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

3. Treat the isolated RNA with an RNase-Free DNase Set (Qiagen, Germany) to eliminate any DNA contaminants according to the manufacturer's protocol.

4. Reverse DNase-free total RNA to cDNA using the OneStep RT-PCR Kit (Qiagen, Germany) according to the manufacturer's protocol.

5. Design the primers for the tick and animal host genes using Beacon Designer 7.0 (PREMIER BioSoft International) (*see* **Note 12**).

6. Optimize PCR runs for all genes to define optimal reaction conditions and efficiencies using cDNA, the designed and control primers, a HotStarTaq Master Mix Kit, and Nuclease-Free Water (*see* **Note 12**).

3.2.2 Cloning and Expression of Both Animal Host and Tick sp. Recombinant Proteins

1. Amplify the tick and animal host genes' ORF and purify the resultant amplicon to be mixed with the TOPO® cloning vector, then incubate for 5 min at room temperature [56, 57] (*see* **Note 13**).

2. Transform the incubated cloning reaction into competent *Escherichia coli* One Shot™ TOP10 cells (Invitrogen) and cultured in LBA media according to the manufacturer's guidelines, and plate it on low-salt LB agar with Zeocin™ (Life Technologies); then screen the resultant colonies by PCR using vector primers according to manufacturer's protocol.

3. Isolate DNA from the positive colonies using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Check the sequence of the putative positive clone plasmid DNA, then analyze the sequence using MacVector with Assembler version 10.0.2.

3.2.3 Purification of Both Expressed Recombinant Proteins

1. Apply a positive colony to a lysis buffer prepared for the Pro-Bond Purification System (Invitrogen, Life Technologies) according to the manufacturer's protocol [57] (*see* **Note 14**).

2. Purify the recombinant proteins for both the animal host and the tick vector using a ProBond™ nickel-chelating resin column. Elute the resultant recombinant proteins using the eluting buffer according to the protein nature and follow the manufacturer's protocol.

3. Analyze the eluted recombinant proteins via SDS-PAGE and western blotting using anti-histidine tag antibodies.

4. Concentrate the eluted protein using Amicon Ultra-15 centrifugation units, then keep the concentrated protein at −20 °C until use.

3.3 Synthetic Peptide Production

1. Synthesize the in silico B-cell epitope predicted peptide, representing the region of the lowest sequence similarity of the tick protein to the animal host protein, commercially according to Rodríguez-Mallon et al. [9] and Mahmoud et al. [57].

2. Conjugate an equimolar mix of the synthetized B-cell epitope peptide to (KLH) before animal immunization.

3.4 Host Immunization Using the Validated Animal Host, Tick Recombinant Proteins, and the Unique Tick Synthetic Peptide

1. Immunize three groups of the animal host with the prepared animal host recombinant protein (s), the tick sp. recombinant protein (s) and the unique tick synthetic peptide(s) according to Rodríguez-Mallon [27] and Galay et al. [28].

2. Vaccinate the animals three times with a three-week interval.

3. Collect the animals' sera before vaccination to be considered as a control negative and according the vaccination schedule.

4. Detect the immunogenicity of the host and tick sp. recombinant proteins and the unique tick synthetic peptide(s) using ELISA and western blotting as previously described by Kurien and Scofield [59], Rodríguez-Mallon [27], Galay et al. [28], and Kasaija et al. [56].

3.5 Analysis of Autoimmune Reaction

3.5.1 In Vitro Autoimmune Analysis

1. Culture the specific animal host cell line in DMEM medium, adding the medium supplements in the appropriate amounts for each cell line type according to the manufacturer's protocol. Incubate the specific animal host cell line in a CO_2 incubator at 37 °C and 5% CO_2 until the culture becomes confluent. Wash the cells with PBS 1x, and prepare the cell culture extract with five freeze–thaw cycles in the cell extract buffer.

2. Homogenize the specific tick specimen with the same extract buffer (see **Note 15**).

3. Centrifuge both extracts (specific animal host and specific tick specimen) at 5000 and 8000 $\times$ g for 30 min, respectively. Collect both supernatants and estimate their total protein contents according to Lowery et al. [58]. Store the host and tick-specific antigens in small aliquots at -20 °C until use [8].

4. Perform a reducing poly-acrylamide gel electrophoresis and western blotting run utilizing 15µL of each specific animal host and specific tick antigen according to the methods and protocol published by Kurien and Scofield [59].

5. Analyze the resultant bands on the nitrocellulose membrane using the ChemiDoc Gel Imaging System (Bio-Rad).

3.5.2 In Vivo Autoimmune Analysis

1. Detect the antinuclear (anticell) antibodies (ANAs) in the animal host sera using either an ANA HEp-2 IFA Kit according to the manufacturer's protocol or the HEp-2 cell line by indirect immunofluorescence cytochemistry [23, 24] (see **Note 16**).

2. Examine the resultant pattern on the slide using an indirect immunofluorescence microscope (see **Note 17**).

3.6 Vaccine Efficacy Evaluation

1. Challenge the animal host groups [animal species will be according to the work plan], with the tick sp. under study, two weeks after the last immunization.

2. Estimate the vaccine efficacy of the host and tick sp. recombinant proteins and the unique tick synthetic peptide(s) immunogens using equation mentioned by Rodríguez-Mallon [27] in case of one host tick spp., and the other equation mentioned by Galay et al. [28], Kasaija et al. [56] in case of three-host tick spp.

4   Notes

1. The BLASTn program is a keystone of bioinformatics that could be used to search and align RNA or DNA genomic sequences which gives information on regions of identical or similar mRNA, expressed sequence tags, noncoding RNA sequences, and genomic DNA sequences.

2. We can predict proteins by analyzing the ORFs, which gives information about possible amino acids that could be retrieved during translation. Additionally, at the NCBI website, the ORF finder identifies all ORFs or possible protein coding regions from six different reading frames (http://vlab.amrita.edu/?sub=3&brch=273&sim=1432&cnt=1).

3. Deep scoring matrices like BLOSUM62 and BLOSUM50 need longer sequence alignments, so they provide very sensitive similarity searches and can produce overextension alignments for non-homologous regions with 20–30% identity [34].

4. MSA algorithms such as in MUSCLE software can readily align apparent conserved motifs with significant improvements in both biologically accurate alignments and computational complexity than CLUSTAL W2 or T-COFFEE programs to overcome the rapid growth of sequence databases of larger protein families. MSA is important for phylogenetic tree estimation, critical residue identification, and secondary structure prediction [35].

5. The value of statistically analyzing the changes in molecular evolution concerns the rate of single nucleotide changes, the genetic basis of speciation, and the ways evolutionary forces influence genomic and phenotypic changes [37, 38].

6. The protein dissimilarity matrix of tick and host amino acid sequences and their orthologs is easily drawn using the integrated R environment software facilities. Using a well-developed, simple, and effective programming language provides effective data handling and storage, coherent and integrated collection of intermediate tools for data analysis, and graphical facilities for data analysis and display [39].

7. The MEME tool is important for searching for novel repeated, un-gapped sequence patterns (motifs) that occur in the DNA or protein sequences for the discovery of new transcription factor binding sites and protein domains. The Motif Alignment and Search Tool can match output motifs encoded in several popular formats and input motifs discovered in their sequence databases [40, 41].

8. The MODELLER program, version 9.22, is used for homology modeling to produce models of protein tertiary structures

by utilizing nuclear magnetic resonance spectroscopy of proteins. Termed satisfaction of spatial restraints relies on an input sequence alignment between the target amino acid sequence to be modeled and a template protein which structure has been solved, by which a set of geometrical criteria are used to create a probability density function for the location of each atom in the protein. The quality of the protein structure is normalized by z-DOPE, an atomic distance-dependent statistical score [42, 43].

9. Linear B-cell epitope prediction could be influenced by different parameters, such as hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity, and the antigenic propensity of polypeptide chains that have been correlated with the location of continuous epitopes and would allow the position of continuous epitopes to be predicted from certain features of the protein sequence. The Chou and Fasman scale is commonly used to predict epitopes via beta-turn prediction (a score above 0.924 is considered to have a high probability of being part of an epitope). The Emini surface accessibility scale is calculated depending on surface accessibility (a score > 1.000 indicates a high probability of the peptide being found on the protein surface). Karplus and Schulz determine the flexibility of protein segments based on the B-factors of 31 protein structures (a score above 0.962 is considered to have a high probability of being part of an epitope). The Kolaskar and Tongaonkar antigenicity scale is based on the physicochemical probabilities of amino acid residues of the targeted protein and their occurrence regularities in known epitopes from other proteins (a score above 1.067 is considered to have a high probability of being part of an epitope). Parker hydrophobicity prediction relies on a hydrophilic scale based on peptide retention times during high-performance liquid chromatography (HPLC) (a score above −0.326 is considered to have a high probability of being part of an epitope). The BepiPred-1.0 linear epitope tool predicts linear B-cell epitopes based on a propensity scale and Hidden Markov Models (a score above 0.350 is considered to have a high probability of being part of an epitope). BepiPred-2.0 uses a random forest algorithm optimized for epitopes and non-epitope amino acids determined from protein crystal structures to predict sequential B-cell epitopes (a score above 0.500 is considered to have a high probability of being part of an epitope) [48, 53].

10. The DiscoTope-2.0 server predicts discontinuous B-cell epitopes from three-dimensional protein structures along the length of a protein sequence using calculation of the solvent-accessible surface area and a novel epitope propensity amino acid score. The final scores are calculated by combining the

propensity scores of residues in spatial proximity and the contact numbers [54, 55].

11. The tissue samples are immediately preserved in RNAprotect Tissue Reagent to stabilize the RNA in its proper structure to preserve the gene expression profile. This reagent permeates the tissues rapidly and prevents the freezing of samples in liquid nitrogen (https://www.qiagen.com/cn/products/discovery-and-translational-research/sample-collection-stabilization/rna/rnaprotect-tissue-reagent/#orderinginformation).

12. To determine the PCR reaction optimum condition, adjust the total volume reactions which contains cDNA, primers, master mix and nuclease-free water as well as, the thermal cycling profile. Additionally, a reference gene, beta-actin must be used for normalization

13. The plasmid (pCR™II-TOPO® vector or pCR™2.1-TOPO® vector) is supplied linearized with TA Cloning® kit whereby, single 3′-thymidine (T) overhangs for TA and Topoisomerase I covalently bound to the vector. Thus, a Taq polymerase adds a single deoxyadenosine (A) to the 3′ ends of PCR products, which when combined with the linearized vector has single, overhanging 3′ deoxythymidine (T), allowing the PCR inserts to ligate efficiently with the vector.

14. The choice of a denaturing, native, or hybrid purification system will be according to the solubility of the protein and the need to preserve biological activity for subsequent applications. If the protein is soluble and you want to retain protein activity, you should follow the native condition. However, the denaturing conditions could be used if the protein is insoluble and you don't need to preserve its protein activity. Additionally, the hybrid protocol could be used if the protein is insoluble, but you want to preserve its protein activity.

15. The specific tick antigen could be filtered by syringe filter cut-off 0.2 μm non-pyrogenic filters, differed according to the origin of the tick specimen [60].

16. All vaccines are suspected to trigger autoimmunity in the animal host through molecular mimicry arising from the homology between the pathogenic antigen component in the vaccine and the specific host protein. Meanwhile, immune system cross-reactivity with the pathogenic antigen may simultaneously trigger the host protein, causing an autoimmune reaction. Autoantibodies are produced by the host's immune system, which then ceases to recognize its self-antigen, leading to the production of autoantibodies [11, 24].

17. Antinuclear antibodies (ANAs) are autoantibodies that produce a nuclear and/or cytoplasmic staining patterns. The nuclear patterns might be homogeneous, speckled (fine and

coarse), peripheral/rim, nucleolar, centromeric, proliferating cell nuclear antigen, nuclear dots, nuclear membrane or grainy diffused, or they can appear in mitotic patterns like mitotic spindle, centrosomes, nuclear mitotic apparatus, mid-body, (centromere protein). While, the cytoplasmic patterns may seem speckled, mitochondrial-like, ribosomal-like, Golgi apparatus, lysosomal-like, cytoskeletal filaments. Among these nuclear patterns, the homogenous, speckled and peripheral pattern are more commonly observed and of clinical importance. The intensity of these fluorescence staining patterns indicates a qualitative scale of positivity, as the fluorescence intensity is related to the antibody concentration and predicts the severity of the case [25].

Acknowledgments

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