Evaluation of bivalent inactivated infectious bronchitis viral vaccine prepared from local isolates

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ABSTRACT:

Infectious Bronchitis (IB) is currently one of the most important viral diseases in poultry flocks all over the world causes huge economic losses in poultry industry due to its effect on broiler growth and its effect on egg production quantity and quality in adult hens. IBV has many serotypes that do not confer cross protection against each other. This study was conducted to prepare bivalent inactivated IBV vaccine by using the local classical and variant isolates (KP279995/2014 and KP279998/2014 respectively) which isolated from different poultry farms in Egypt as previous surveillance study during IBV outbreak from Al-Sharkia and Al-Qalubia governorates. The locally prepared vaccine formulated by using formalin for inactivation and Montanide ISA71 RVG as oil adjuvant. The prepared bivalent inactivated (IBV) vaccine was tested for sterility, safety and potency, the efficacy of the prepared vaccine was applied in specific pathogen free chicks (SPF) for monitoring the antibody titers by using of Enzyme Linked Immuno Sorbent Assay (ELISA) post vaccination with estimation of shedding parameters by Real-Time RT-PCR in challenging birds with $10^{4.5}EID_{50}$/dose challenge IBV strains (classical and/or variant). The results revealed that the prepared vaccine free from any bacterial or mycotic contamination also safe after double dose inoculation in SPF chicks. Vaccinated birds showed a good percent of protection with elevation of antibody titers in comparison with unvaccinated ones. This confirms that under field condition, poultry industry can be protected from IB disease if using locally isolates in preparing of

inactivated vaccine which reduce the economic losses caused by IB infection viruses in Egypt.

Key words: Infectious bronchitis virus, Real-Time RT-PCR, Inactivated vaccine.

1. Introduction:

The avian infectious bronchitis virus (IBV) is a highly contagious pathogen of commercial chickens with a predilection for the upper respiratory tract and it cause reduction in production, in addition to causing disease in kidneys resulted in nephritis .In adult, IBV effect on reproductive tract and produce irreparable damage to oviduct and production of abnormal eggs. (**Arthur Sylvester *et al*., 2005; Cook *et al*., 2012; Jackwood, 2012; Jackwood and De Wit, 2013**).

IBV is a gammacoronavirus, family coronavirdae, order Nidovirales (**Cavanagh and Naqi, 2003**). The virus is a single-stranded, positive sense, 27 kb RNA genome that encodes many nonstructural proteins involved in replication, three major structural proteins (spike (S), envelope (E), membrane glycoprotein (M) involved in virion formation and a protein involved in genome packaging nucleocapsid (N) (**Cavanagh, 2007**). The S glycoprotein is cleaved into two subunits, S1 and S2, of these proteins, S1 is often regarded as the most significant due to its role in host cell binding and neutralizing epitope presentation (**Promkuntod *et al.*, 2014**).

IBV has a constant threat to the poultry industry although vaccination has been considered to be the most cost effective approach to controlling IBV infection (**Meeusen *et al*; 2007**). Even though the poultry industry extensively vaccinates against IBV, immunization programs now seem to be ineffective due to the different antigenically serotypes and newly emerged variants from field chicken flock which sometimes vaccine breaks (**Gelb *et***

***al.*, 1991**) . Hence, effective diagnostic tools are needed to differentially diagnose IB infections in field and to identify different serotypes and variants. It gives us an insight, as to whether the vaccine virus is able to give proper protection or not and this information is crucial as new strategies have to be designed and developed both in diagnosis and control of the disease (**Arthur Sylvester *et al*., 2005; Jordan, 2017**). Emergence of new "variant" strains require rapidly preparation of inactivated autogenous vaccines for controlling IB in laying birds without the risks of using a live variant vaccine that could spread and potentially produce the disease. Inactivated variant vaccines may offer better protection against challenge with the virulent variant IBV than inactivated vaccines containing standard serotypes such as Mass and Conn **(Ladman *et al* ., 2002; Jackwood, 2012)**.The aim of the present study was directed to carry out preparation of bivalent inactivated IBV vaccine from locally isolated IBV strains (classical and variant) and study the quality assurance of this locally prepared inactivated IB vaccine.

2. MATERIALS AND METHODS:

2.1. IBV strains:

IBV strains were previously isolated from different localities by (**Reda *et al*; 2015**), (Chicken / Egypt KP279995 / VACSERA / 2014) (classical) and (Chicken / Egypt KP279998 / VACSERA / 2014) (variant) .The two strains were propagated in SPF-ECE and tittered with infectivity titer $10^{8.5}EID_{50}$ / dose as viral seed for preparation of bivalent inactivated IB vaccine and challenge dose was adjusted to be $10^{4.5}$ EID_{50} /ml, the titration was calculated according to (**Reed and muench, 1938)**

2.2. Experimental hosts:

2.2.1. Embryonated Chicken Eggs (SPF-ECE):

Fertile specific pathogen free embryonated chicken eggs (SPF– ECE) were purchased from the SPF egg farm, Kom Oshim, EL-Fayoum Governorate, Egypt. The eggs were used for propagation and titration of the seed IB viruses and assurance of complete virus inactivation of tested inactivated IB vaccine.

2.2.2. SPF chicks:

Total number of 200, one day-old SPF chicks were purchased from SPF poultry project, Kom Oshim, EL-Fayoum Governorate, Egypt. All birds were housed in a separated negative pressure –filtered air isolators.

2.3. Enzyme –linked Immunosorbent Assay (ELISA) kit:

Serum samples were tested for detection of antibodies to IBV after vaccination by using ELISA Kit which was obtained from Biocheck poultry immune assays. IBV antibody test kit (CK119): Serial No. F69371 product code: 6020 used in this study, all procedures were conducted according to manufacturer's instructions.

3.4. Real-time Reverse transcriptase chain reaction (RRT-PCR):

Tracheal and kidney samples were collected and stored at -20°C till used , it were tested for detection and titration of IBV in both vaccinated and un vaccinated control groups at 3,5,7 days post challenge . Extraction of viral RNA carried out by QIA amp Viral RNA Mini Kit (QIAGEN) with catalogue No. (52904). All procedures were conducted according to manufacturer's instructions. Preparation of master mix was conducted using Quanti Tect probe RT-PCR with catalogue no. (204443) provide accurate real-time quantification of RNA targets, Primers and probes used were supplied from Metabion (Germany) as shown in table (1). With the

following cycling conditions: reverse transcription at 50°C for 30 min , primary denaturation at 95°C for 15 min; 40 cycles of 94°C for 15 sec; 60°C for 45 sec (annealing +extension).The numbers of viral genome copies were quantified in a TaqMan® real time RT-PCR targeting IBV gene.

Virus	Gene	Primer/ probe sequence 5'-3'	Ref
IB	N	AIBV-(Forward) ATGCTCAACCTTGTCCCTAGCA	**Meir *et al*., 2010**
		AIBV-(Reverse) TCAAACTGCGGATCATCACGT	
		AIBV-TM (Probe) [FAM]TTGGAAGTAGAGTGACGCCCAAACTTCA [TAMRA]	

Preparation of an experimental batch of bivalent inactivated IBV vaccine:

1. Propagation and titration of IB viruses in SPF-ECE: (OIE, 2018)

Two field isolates (KP279995, Classical) and (KP279998, Variant) were propagated in the allantoic fluid of SPF-ECE at 9-11 day old and incubated at 37C with 80% humidity, after 72 hrs of inoculation, allantoic fluids were harvested from about 12 passages of inoculation. The second step was titration in SPF-ECE (tenfold serial dilution in sterile physiological saline, 0.1ml of the virus suspension dilution was inoculated in to the allantoic sac of each of 10 days old SPF-ECE and incubated at 37°C with daily candling.

2. Inactivation of the propagated IBV:

The harvested infected allantoic fluid was treated with Formalin 37 % (Sigma- Germany) at a final concentration of 0.1 % with continuous stirring during inactivation process according to (**Beard, 1989**). Samples from the inactivated virus, should be tested for completion of inactivation

by passage (at least 2 blind passages) in to 9-11 days old, ECE (0.1 ml/egg) via the allantoic cavity route and examined for 3 days.

3. Preparation of the vaccine emulsion:

It was prepared as water in oil emulsion (W/O) using Montanide™ ISA71 RVG (SEPPIC, Paris La Defense. 92806 Puteaux, France batch No. T21931) at a ratio of 30/70 (v/v) according to the standard protocol of SEPPIC for manufacture instruction **(Ben Arous et al., 2013).**

Evaluation of the prepared bivalent inactivated IBV oil emulsion vaccine:

Testing of quality control of the prepared bivalent inactivated IBV vaccine including sterility, safety and potency tests which were carried out according to (**OIE, 2018**) and (**Egyptian standard regulation for veterinary Biologics, 2017**).

1-Sterility test:

Media used for sterility test:

1-Nutrient agar media for detection of aerobic bacterial contamination.

2-Thioglycolate broth for detection of anaerobic bacterial contamination.

3-Saburaoud dextrose agar media for detection of fungal contamination.

It was applied to confirm that the prepared bivalent IBV inactivated vaccine was free from bacterial and fungal contamination. Samples from the tested vaccine inoculated into nutrient agar and thioglycolate broth media then incubated at 37°C for detection of any bacterial contamination also the sample were cultured on Saburaoud dextrose agar media and incubated at 25°C for 14 days to detect any fungal contamination. The inoculated media were inspected daily for any possible growth.

2-Safety test:

Forty SPF chicks aged 3 weeks old were divided into two equal groups (20 chicks/ group), the first group was inoculated S/C with double the field dose

of the tested vaccine and the second group were kept unvaccinated as control. Birds in both groups were observed for any signs of local reaction or appearance of any clinical signs of IBV and post mortem examinations to detect any pathological lesions for 15 days.

3-Potency of the prepared vaccine:

A total of 160 SPF one day old chicks were used to evaluate the potency and efficacy of locally prepared bivalent inactivated IB vaccine, they were housed in brooder units within isolation facilities. At 21 days of age, the chicks were divided into 6 equal groups (25 birds/group) and one control negative group (10 birds) as in table (2). Frist 3 groups vaccinated S/C with field dose (0.5ml) of prepared vaccine / bird, the other 3 groups were left without vaccination. Blood samples were taken weekly for serological analysis of IB antibody level using ELISA test. Three weeks post vaccination, the vaccinated groups (G1, G2, G3) and unvaccinated control groups (G4, G5, G6) were subjected for challenge test with IBV strains and group (7) kept as control (-Ve) unvaccinated unchallenged. Each bird received a dose of 0.1 ml oculonasal rout of IBV strains ($10^{4.5}$ EID_{50} /ml) and observed for 15 days after challenge for clinical signs and postmortem lesion in trachea and kidney which were collected at 3^{rd}, 5^{th} and 7^{th} days post challenge to determine the virus shedding using (RRT-PCR).

Table (2): Challenged vaccinated groups and unvaccinated control groups

Group No	No of birds	Vaccination at 3 weeks old	Challenged at 6 weeks old
Group 1	25	bivalent inactivated IBV vaccine	classical strain (KP279995)
Group 2	25	bivalent inactivated IBV vaccine	variant strain (KP279998)
Group 3	25	bivalent inactivated IBV vaccine	Two IBV classical and variant strains.
Group 4	25	unvaccinated control (+)	classical strain (KP279995)
Group 5	25	unvaccinated control (+)	variant strain (KP279998)
Group 6	25	unvaccinated control (+)	two IBV classical and variant strains
Group 7	10	unvaccinated control (-)	unvaccinated unchallenged

3. Results:

3.1. Results of Propagation and titration of IBV strains separately from each other on specific pathogen free embryonated chicken eggs (SPF-ECEs):

Local isolate IBV strains (Chicken / Egypt KP279995 / VACSERA / 2014) (Classical) and (Chicken / Egypt KP279998 / VACSERA / 2014) (variant) were propagated in 9-10 day old, SPF-ECE for 12 passages , allantoic fluids were harvested and tested for sterility. The virus was titrated using infectivity titration on SPF eggs, the virus titer was calculated as titer $10^{8.5}$ EID_{50} /ml. This virus titer was used as the seed for vaccine preparation.

3.2. Results of Inactivation of IBV strains separately from each other by 0.1% formalin with ensured completion of inactivation:

The harvested allantoic fluid of SPF-ECEs after propagation and titration of the viruses are inactivated by using 0.1% formalin solution. IBV was proved to be completely inactivated as indicated by absence of any pathological lesions, and/or deaths of inoculated embryos being inoculated in 9 days old, SPF-ECEs through the allantoic sac and candled daily for 6 days.

3.3. Quality control tests of the prepared bivalent inactivated IBV vaccine:

3.3.1. Results of sterility test:

It was found that the prepared bivalent IB vaccine was sterile as it is free from any bacterial and fungal contaminants

3.3.2. Results of safety test:

For safety examination of the prepared bivalent IB vaccine gave neither local nor systemic reactions and no mortalities were observed among inoculated chicks indicating safety of the prepared vaccine.

3.3.3. Results of potency test:

1. Monitoring of serum antibody titer in chicks vaccinated with inactivated bivalent IBV vaccine using ELISA test:

In order to estimate the antibody titers in chicken groups vaccinated with inactivated bivalent IB vaccine, ELISA test was used. The minimum positive level serum is equal or more than 833 in ELISA antibody titers for inactivated vaccine according to kit manufacture.

The mean antibody titers for vaccinated groups estimated all over 3 weeks revealed that It was noticed that chicks vaccinated with inactivated bivalent IB vaccine showed significant increase at 2^{nd} week post vaccination (WPV) (1152) and increased to reached (2789) at the 3^{rd} WPV in comparison with

control (SPF chicks non-vaccinated kept as negative control) as shown in table (3) and figure (2).

Table (3): Mean antibody titers of IBV vaccine using ELISA test:

Group/ weeks	Vaccinated groups	Control groups
Before vaccination	12.73±78.3	11.06±23.1
1 week post vaccination	577.3±14.6	19.40±9.07
2 week post vaccination	1152.7±1.76	30.66±29.6
3 week post vaccination	2789.7±58.4	38.00±30.5

N.B: The minimum positive serum level is to be equal or more than 833 according to kit manufacture.

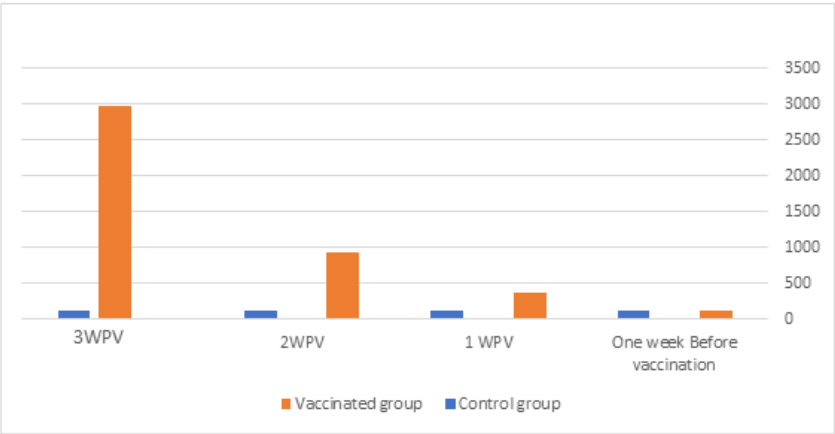


Figure (2): GMT of IBV ELISA reading in both vaccinated and unvaccinated control groups before and after vaccination

2. Results of IB vaccine efficacy:

Results of challenge test in vaccinated groups with locally prepared vaccine showed that challenging of immune vaccinated chicks were

protected against IB disease for 15 days post challenge with 92% protection with comparison to the control group (40% protection). Tracheal and kidneys samples were collected at 3rd , 5th and 7thday post challenge from vaccinated groups (G1 , G2 , G3) , control positive groups (G4 , G5 , G6) and control negative group (G7) for post mortem examination as shown figure (3). The results for determination of viral shedding by using RRT-PCR at 3th, 5th and 7th days post challenge are shown in table (5) and figure (4).

3.4. Parameters for challenge experiment:

3.4. a. Clinical signs rate

All chickens wereexamined daily for clinical signs of IBV infection such as coughing, sneezing, nasal discharge, head shaking, lethargy and conjunctivitis .the clinical signs were recorded according to scoring index as in table (4)

Table (4): Clinical signs score system of infected chicks:

Clinical signs	Score
No clinical signs	0
Lacrimation, slight head shaking and watery faces	1
Lacrimation ,presence of nasal exudates, depression and watery faces	2
Strong (lacrimation ,presence of nasal exudates, depression) and severe watery faces	3

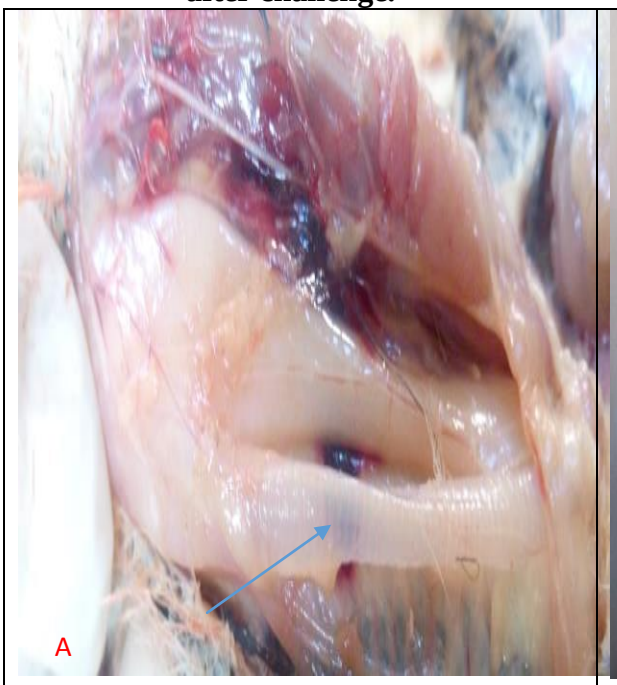
Scoring index according to (**Avellenda et al., 1994), (Wang and Huang, 2000)**

Table(2): Recording clinical signs rate after challenge with challenge strains in vaccinated and unvaccinated control groups:

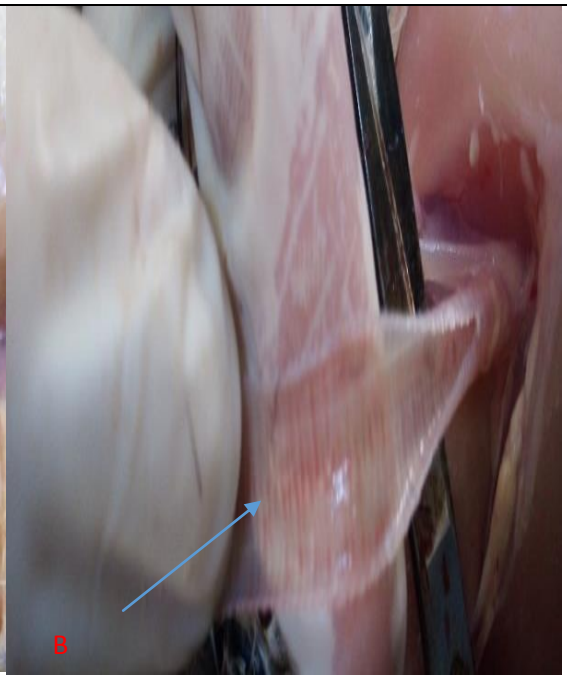
Group No	Days post challenge and number of dead birds										Total NO	Total %	Protection %
	1	2	3	4	5	6	7	8	9	10-15			
G1	-		1	-	-	-	-	-	-	-	1/25	4	96
G2	-		1	-	1	-	-	-	-	-	2/25	8	92
G3	-	-	-	1	-	-	1	-	-	-	2/25	8	92
G4	-		2	-	5	-	-	-	1	-	8/25	32	68
G5	-		4	2	1	3	1	-	1	-	12/25	48	52
G6	-		5	3	2	1	1	1	1	1	15/25	60	40
G7	-	-	-	-	-	-	-	-	-	-	0/10	0	100

N.B: Birds in groups (G1, G2, G3) have clinical signs score (1), however in group (G4, G5, G6) have clinical signs score (3)

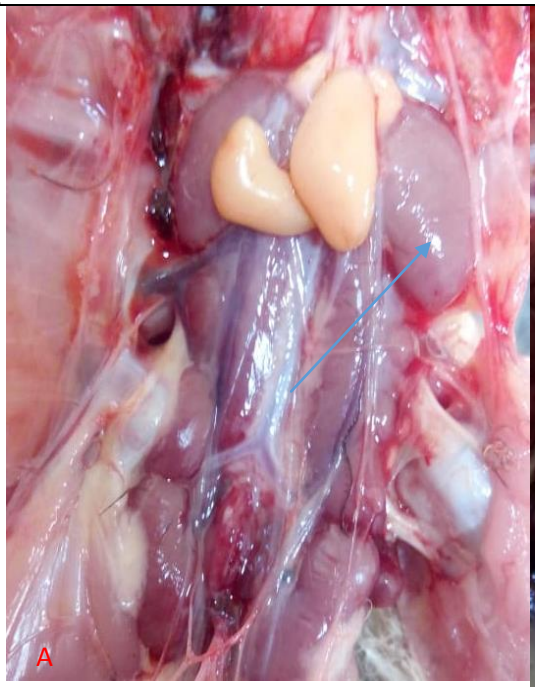
Figures (3): Post mortem examination in vaccinated and unvaccinated chickens after challenge.



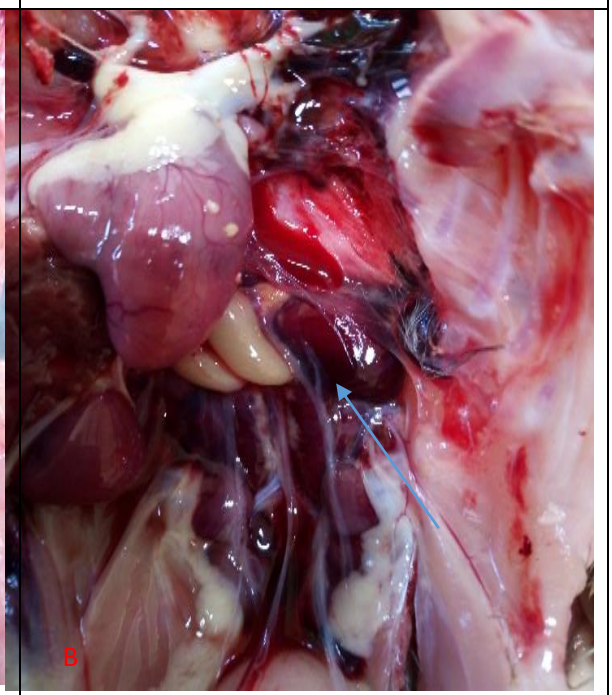
A: Trachea of vaccinated challenged groups after 5 days from challenge showed normal anatomical structure with transparent appearance



B: Trachea of unvaccinated challenged groups after 5 days from challenge showed mucoid tracheitis



A: Kidney of vaccinated challenged groups after 7 days from challenge showed normal anatomical structure.



B: Kidney of unvaccinated challenged groups after 7 days from challenge showed severe congestion of renal tubules

Table (5): The results of real time PCR of shedding IBV from both vaccinated, unvaccinated challenged birds with IBV strains (classical and /or variant)

Day post challenge	Group	Result	Cycle threshold (Ct)	Titer (EID50/ml)
3 days	Group 1	Positive	22.98	1.878×10^2
	Group 2	Positive	22.87	2.028×10^2
	Group 3	Positive	20.98	7.602×10^2
	Group 4	Positive	19.43	2.247×10^4
	Group 5	Positive	19.85	1.675×10^4
	Group 6	Positive	20.10	1.406×10^4
	Group 7	Negative	No Ct	(-)
5 days	Group 1	Positive	21.08	1.089×10^2
	Group 2	Positive	21.75	1.438×10^2
	Group 3	Positive	24.11	4.525×10^2
	Group 4	Positive	18.75	3.614×10^4
	Group 5	Positive	18.21	5.271×10^4
	Group 6	Positive	18.34	4.813×10^4
	Group 7	Negative	No Ct	(-)
7 days	Group 1	Negative	No Ct	(-)
	Group 2	Negative	No Ct	(-)
	Group 3	Negative	No Ct	(-)
	Group 4	Positive	18.20	5.308×10^3
	Group 5	Positive	18.02	3.020×10^3
	Group 6	Positive	18.76	6.589×10^3
	Group 7	Negative	No Ct	(-)

N.B: The shedding of IBV decrease from 3rd to 5th day post challenge in vaccinated birds in groups 1, 2, 3 in comparison with control (+ve) groups

4, 5, 6 . The birds at 7th day, there are no shedding of IBV which it indicated the protection against challenge virus.

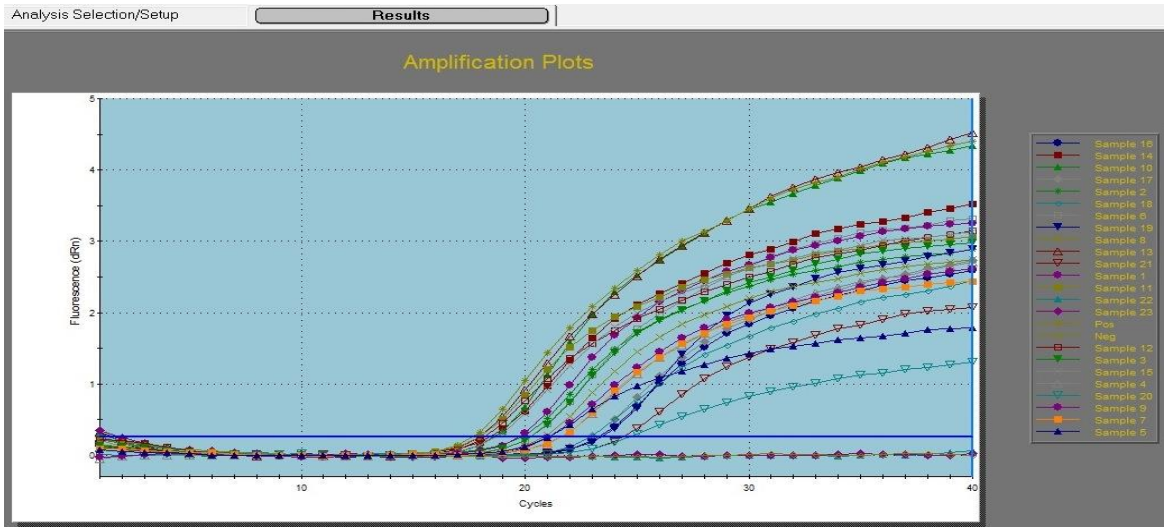


Figure (4): Results of Real-time PCR for detection of IBV in both vaccinated and unvaccinated groups after challenge

4. Discussion

Infectious bronchitis (IB) is a highly contagious viral disease of chickens. It is possibly the most economically important viral respiratory disease of chickens in regions where there is no highly pathogenic avian influenza virus(AIV) or velogenic Newcastle disease virus and is found everywhere that commercial chickens are kept. While initially a respiratory disease, the virus also affects the female reproductive tract, causing loss of production and poor egg quality. Some strains have a predilection for the kidney of young chickens, resulting in nephritis that can cause significant mortality (**Cook *et al*; 2012**).

Serotypic evolution in IBV are associated primarily with changes in the sequence of the S1glycoprotein, which contains regions associated with virus attachment and epitopes that induce production of neutralizing antibodies (**Cavanagh *et al*., 1988**). Different serotypes, subtypes, and

variants of IBV are thought to be generated by nucleotide point mutations, insertions or deletion (**Jia *et al.*, 1995**), which are responsible for the problem of outbreaks of IB disease in previously vaccinated chicken flocks.

The present study was an attempt for preparation of bivalent inactivated oil emulsion IBV vaccine from locally isolated IB viral strains as result from molecularly identified IBV strains from surveillance study during outbreak at 2014. The trail was target for production of bivalent inactivated vaccine containing both classical strain related to H120 (KP279995/2014) and variant strain like IS1494 (KP279998/2014), this combination for covering the antigenic spectrum of isolates in particular region and it was able to providing sufficient protection against the prevailing field strains that can be homologous to the most important dominant field strains which could induce broad cross protection against many strains. In addition to evaluation and application of quality control parameters of the locally prepared vaccine to be fit for use in Egyptian poultry farms. Locally prepared vaccine was propagated in SPF eggs (9-10) days, the allantoic fluid is collected after 72 hrs post inoculation for 12 passages as described by (**Clarke *et al.*, 1972**), (**Jackwood *et al.*, 1992**), (**CFR, 2017**) and (**OIE, 2018**).

The results of titration was judged according to the parameters of (**CFR, 2017**) in which IBV titer must be not less than $10^{3.5}$ EID_{50} / dose. So the prepared master seed of IB strains were satisfactory with $10^{8.5}$ EID_{50} /ml. The results of master seed vaccine is free from bacterial and fungal which judged according to parameters of (**European pharmacopoeia, 2013**) in which the master seed used for preparation of inactivated vaccine must be sterile and free from any contamination.

The locally prepared inactivated vaccine considered to be safe according to the requirement of (**OIE, 2018**) if inoculation with double dose in 3 weeks old SPF chicks and gave no serious signs or deaths during the observation period (21 days post inoculation) . Results for Eco toxicity test agree with the (**European pharmacopoeia, 2013**).

In studying of potency effect of the vaccine was applied in vivo by measuring of ELISA post vaccination and estimation of viral shedding by RRT-PCR post challenge for detection of vaccine efficacy. The vaccinated birds gave GMT 382, 933 and 2969 in 1st, 2^{nd} and 3^{rd} week post vaccination in comparison with unvaccinated negative control. The ELISA technique is a sensitive serological method and gives earlier reaction for antibody titers than other tests as (**Mockett and Darbyshire, 1981**). The protection % for vaccinated birds when challenge against homologous classical / variant either separated or combined gave higher protection rate (92%) with vaccinated challenged groups with comparison in shedding rate of RRT-PCR reading in control (+ ve) groups either in 3 , 5 and 7 days post challenge. This results were agree with (**OIE, 2018**), (**CFR, 2017**). That indicated the effect of prepared vaccine in control infectious bronchitis in Egyptian field.

5. Conclusion

Finally, virulent IBV continues to be endemic in Egypt despite the application of billions of doses of live and inactivated IB vaccines. So, the selection of vaccinal antigens must be done on the basis of the cross-protection studies. The locally prepared IB viral vaccine from both classical and variant field strains is safe, potent and induce high levels of antibody titers, in addition to giving a high protection in vaccinated chickens and

recommended for using in prevention of IBV circulating in the poultry farms.

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