

The utility of polymerase chain reaction for diagnosis of lumpy skin disease in cattle and water buffaloes in Egypt

S.S.A. Sharawi⁽¹⁾ & I.H.A. Abd El-Rahim⁽²⁾

(1) Department of Virology, Faculty of Veterinary Medicine, Banha University, 13736 Moshtohar, Banha, Egypt

(2) Department of Animal Medicine, Faculty of Veterinary Medicine, Assiut University, 71526 Assiut, Egypt

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Summary

An outbreak of lumpy skin disease (LSD) occurred among cattle and water buffaloes in Egypt in 2006. Polymerase chain reaction (PCR) and the agar gel precipitation test (AGPT) were compared. Eight of ten (80%) tissue specimens from diseased cattle were positive with AGPT while 100% were positive with PCR. Of ten tissue specimens from diseased water buffaloes, 70% were positive with AGPT while 100% were positive with PCR. Ten milk samples were obtained from diseased water buffaloes; PCR detected nucleic acid of LSD virus (LSDV) in 50% while AGPT failed to detect LSDV antigen. Water buffaloes are susceptible to LSDV infection. The clinical signs of LSD were less severe in water buffaloes, but the virus was excreted in their milk. Diagnosis of LSD outbreaks by PCR will facilitate rapid application of control measures. Mass vaccination should be applied in both cattle and water buffaloes in Egypt using an effective specific vaccine against LSD, such as the attenuated Neethling strain vaccine or a recombinant vaccine.

Keywords

Agar gel precipitation test – Cattle – Chorio-allantoic membrane – Egypt – Lumpy skin disease – Milk sample – Outbreak – Polymerase chain reaction – Skin nodule – Water buffalo.

Introduction

Lumpy skin disease (LSD), along with sheep pox and goat pox, is one of the most serious poxvirus diseases of livestock. These diseases are caused by viruses of the genus *Capripoxvirus* within the subfamily *Chordopoxvirinae*, family *Poxviridae* (4). Lumpy skin disease was described as a clinical manifestation, termed 'pseudo-urticaria', in Zambia in 1929 (28). It appears to have spread northwards from South Africa after 1940 (17). By 1943, when it was recognised as an infectious disease (39), it had reached Botswana (19). In 1957, an outbreak of LSD was recorded in Kenya, associated with an outbreak of sheep pox (40). In 1970, LSD spread north into Sudan; by 1974 it had spread west as far as Nigeria, and in 1977 it was reported from Mauritania, Mali, Ghana and Liberia. Another epizootic of LSD between 1981 and 1986 affected

Tanzania, Kenya, Zimbabwe, Somalia and Cameroon, with reported mortality rates in affected cattle of 20%. However, the true extent of this epizootic was not clear, and it probably affected a large area of central Africa. Capripoxviruses have the potential to become emerging disease threats because of global climate change and changes in patterns of trade in animals and animal products (5).

In Egypt, LSD virus (LSDV) was isolated for the first time from cattle in two disease outbreaks in 1988. The virus was detected in a pooled sample from the first outbreak (in Suez) and was isolated subsequently from the second outbreak (Ismalia). The capripoxviruses were identified as LSDV by neutralisation with specific antiserum and by their ability to produce generalised LSD in experimentally inoculated cattle (21). In 1988 and 1989 an outbreak of confirmed LSD occurred among cattle in Egypt (3). In

1998, an outbreak of LSD was reported among cattle in El-Menia Governorate in Upper Egypt (1). Recurrent LSD outbreaks among cattle were recorded in 2006 in Banisuef, Behera, Ismalia and New Valley provinces in Egypt (42).

The first report of LSD outside Africa was described in Kuwait in 1986, where 642 cases were reported in cattle (31). Since then cases have been confirmed or suspected in the United Arab Emirates and Republic of Yemen (41). In 1992, LSDV infection was also reported in Saudi Arabia (18). Lumpy skin disease must be considered to have the potential to become established outside Africa. An outbreak was reported in Bahrain in 1993 but was not confirmed by virus isolation. An outbreak occurred in 2000 in cattle imported into Mauritius; the diagnosis was confirmed by electron microscopy (43).

Lumpy skin disease is of economic importance because it causes reduced production, particularly in dairy herds. It also causes damage to the hide (43). In experimentally infected cattle, the skin nodules involve both the dermis and the epidermis and may exude serum initially, but over the following two weeks necrotic plugs develop that penetrate the full thickness of the hide (13).

The causative agent of LSD is a capripoxvirus, as mentioned above. The prototype strain of LSD is the Neethling virus (2). The LSD virus is one of the largest viruses known (170–260 nm by 300–450 nm) (30). There is only one serotype of LSDV, and it is very closely related serologically to the virus of sheep and goat pox (SGP), from which it cannot be distinguished by routine virus neutralisation or other serological tests (7). Restriction endonuclease studies of capripoxviruses indicate that LSDV strains have a greater than 98% sequence homology with a Kenyan strain (O 240/KSGP) of SGP virus (SGPV) (25).

Transmission of LSDV is thought to be predominantly by insects; transmission by natural contact in the absence of insect vectors is inefficient. There may be failure of the virus to infect a whole group of animals, depending on vector prevalence (43). It has been suggested that transmission of LSDV between animals by direct contagion is extremely inefficient, and that parenteral inoculation of virus is required to establish infection (9). The high proportion of animals with generalised disease following intravenous inoculation implies that naturally occurring cases of generalised LSD may follow spread by intravenously feeding arthropods. While epizootic spread of LSD has occurred in Kenya, most cases are of a sporadic nature and are thought to be the result of accidental contacts with a maintenance host (14). It has been found that female *Aedes aegypti* mosquitoes are capable of mechanical transmission of LSDV from infected to susceptible cattle. Mosquitoes that had fed upon lesions of

LSDV-infected cattle were able to transmit the virus to susceptible cattle over a period of 2 to 6 days after infective feeding. It has been suggested that mosquito species are competent vectors of LSD (12).

Lumpy skin disease is a pox disease of cattle that is characterised by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death (43). Following experimental infection with LSDV in cattle, the animals developed fever and skin nodules, although the extent of infection varied among the animals. Skin nodules remained visible until the end of the experiment on day 42 post inoculation (5).

Lumpy skin disease virus can be grown on the chorio-allantoic membrane (CAM) of embryonated chicken eggs. Maximum yield of LSDV was obtained in the CAM of 7- to 9-day-old embryos inoculated at 33.5°C and 35°C for 5 to 6 days (38). The virus neutralisation test is the most specific serological test, but because immunity to LSD infection is predominantly cell-mediated, the test is not sufficiently sensitive to identify animals that have had contact with LSDV and have developed only low levels of neutralising antibody. The agar gel immunodiffusion test and indirect immunofluorescent antibody test are less specific because of cross-reactions with antibodies to other poxviruses (43).

In epizootics, a diagnosis of LSD is made on the basis of the characteristic lesions in acute severe cases. Diagnosis of mild or atypical cases, especially under enzootic conditions, is difficult and requires laboratory confirmation to demonstrate the virus. Cultures are examined for the characteristic inclusions, with final identification of LSDV being made by the neutralisation test (27). Polymerase chain reaction (PCR) has been shown to be the method of choice for the diagnosis of SGP directly from clinical specimens (29).

The current report describes a natural occurrence of LSDV infection in cattle and water buffaloes in Egypt; the animals showed typical clinical signs of the disease. The excretion of LSDV in the milk of infected buffaloes was observed. In addition, the utility of PCR was investigated for the detection of the DNA of LSDV in clinical specimens such as tissue specimens and milk samples.

Materials and methods

History, epizootiological aspects and clinical examinations

At the beginning of the summer season of 2006, a high incidence of skin lesions in the form of nodules was

observed among cattle and water buffaloes on the farm of the Faculty of Veterinary Medicine, Moshtoher, Kalubia province, Egypt. This farm contained 37 cattle and 60 water buffaloes at the time of sample collection. Cattle and water buffaloes are housed together on this farm. Clinical examination of the affected animals revealed typical clinical signs of LSD, and an outbreak of LSD among cattle and water buffaloes on the farm was suspected.

Samples

Ten tissue specimens (skin nodules), one per affected animal, were collected from ten cattle. Ten tissue specimens (skin nodules) from ten buffaloes, as well as ten milk samples from the same diseased buffaloes, were also obtained from the affected herd. In addition, ten milk samples were collected randomly from the bulk milk tank on the farm during the investigation of the disease. The samples obtained were used for virological and molecular diagnosis.

Sample preparation

Following the method of Burlison *et al.* (8), a 10% suspension was prepared from each skin nodule and each sample of milk. In addition, sedimented pelleted milk samples were prepared in phosphate-buffered saline (PBS) (Sigma, USA; lot no. 068K8202; 0.04 M, pH 7.2–7.6) with added antibiotics (penicillin 1,000 IU/ml and neomycin sulphate 100 IU/ml). The samples were used for the agar gel precipitation test (AGPT), chicken embryo inoculation and PCR.

Virus isolation and titration

According to the method of Burlison *et al.* (8), 0.1 ml of each prepared sample was inoculated onto CAM. The harvested CAM were examined for any changes. After three passages, the isolated virus was titrated in embryonated chicken eggs in accordance with the method of Reed and Muench (33). Tenfold serial dilutions from pools of harvested positive inoculated CAMs were used, i.e. 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} . Each of these dilutions was inoculated into seven embryonated chicken eggs. Any lesions that occurred up to 7 days post inoculation were recorded.

Detection of lumpy skin disease virus antigen by agar gel precipitation test

The AGPT was performed for the detection and identification of LSDV in each sample and its inoculated CAMs individually. The technique was applied in

accordance with the method of Tantawi and Borzovich (35). Hyperimmune serum prepared in rabbits was generously supplied by the Veterinary Vaccine and Serum Research Institute, Abbasia, Cairo, Egypt.

Detection of lumpy skin disease virus by polymerase chain reaction

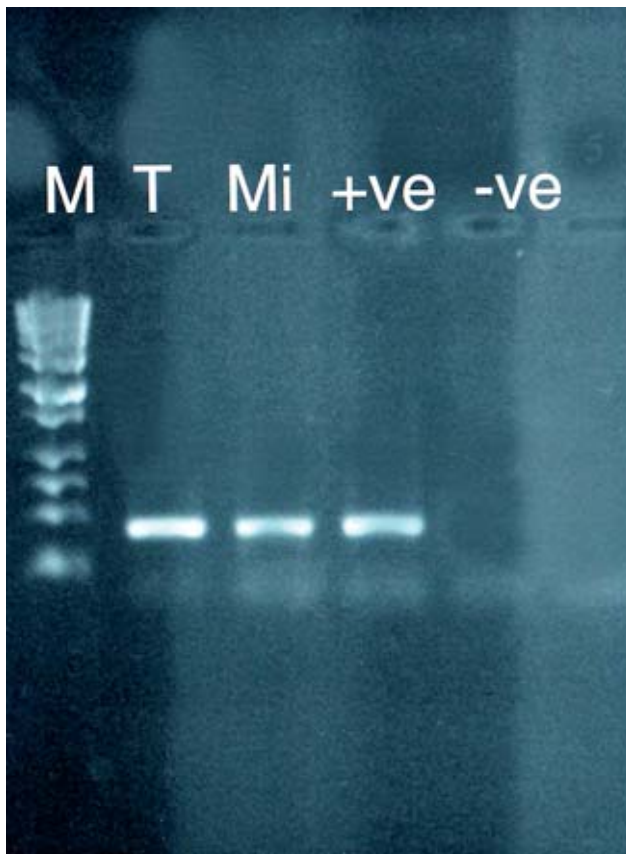
The DNA from each sample was extracted and purified as described by Sambrook *et al.* (34). The DNA extracted from each sample was amplified using the protocol published by Ireland and Binopal (22). Briefly, each reaction mixture (50 μ l) contained 250 ng of total DNA, 2 mM $MgCl_2$, 50 pmol of each primer (forward primer 5'-TTTCCTGATTTTCTTACTAT-3' and reverse primer 5'-AAATTATATACGTAAATAAC-3'), 200 μ M of each dNTP and 2 U of DNA polymerase (Biotool, USA) in a reaction buffer (10 \times) containing 75 mM Tris-HCl (pH 9), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$ and 0.001% bovine serum albumin. Amplification was carried out in an MJ Thermal Cycler (MJ Corporation, USA), programmed to perform a denaturation step of 95°C for 5 min, followed by 40 cycles consisting of 1 min at 94°C for denaturation, 1 min at 50°C for primer annealing and 1 min at 72°C for extension. The last extension step was 10 min longer. A 10 μ l volume of PCR products was mixed with 2 μ l gel loading buffer (Sigma-Aldrich) and electrophoresed in 1% agarose gel containing 1 μ g/ml ethidium bromide in Tris-acetate buffer (0.04 M Tris-acetate and 0.001 M EDTA, pH 8).

The resulting DNA fragments were visualised by ultraviolet transillumination and photographed in accordance with the method of Sambrook *et al.* (34). A visible band of the appropriate size, 192 bp, was considered a positive reaction (Fig. 1). A reference LSDV isolate propagated on a Madin–Darby bovine kidney cell line was obtained from the Virology Department, Animal Health Research Institute, Dokki, Giza and used as a positive control.

Results

Epizootiological aspects and clinical signs

In 2006, an outbreak of LSD started at the beginning of the summer season on the farm under investigation and in the surrounding area. The disease affected cattle and water buffaloes of different ages. Housing of cattle and water buffaloes together facilitated the spread of the disease among these animals. The most noticeable clinical signs were high fever and inappetence, followed by the sudden appearance of firm skin nodules and a drop in milk yield, as well as enlargement of the superficial lymph nodes.



M: marker
 Mi: milk sample
 T: tissue sample
 +ve: positive control
 -ve: negative control

Fig. 1
Detection of DNA of lumpy skin disease virus by polymerase chain reaction (agarose gel electrophoresis of the polymerase chain reaction products)



Fig. 3
Lumpy skin disease lesions all over the body of an infected cow



Fig. 4
Skin nodules in a calf affected by lumpy skin disease



Fig. 2
Water buffalo suffering from skin nodules (lumps) on the left side due to lumpy skin disease virus infection



Fig. 5
Skin nodules of varying sizes in a cow suffering from lumpy skin disease

In some cases, the skin nodules appeared all over the animal's body (Figs 2, 3, 4 and 5). The number of skin nodules per affected animal was greater in cattle than in water buffaloes, and in general the clinical disease was less severe in water buffaloes.

Virological and molecular diagnosis of lumpy skin disease

Eight out of ten (80%) tissue specimens from cattle were positive with AGPT, while 10/10 (100%) were positive with PCR. In addition, of the CAM that were inoculated with the prepared tissue specimens, 8/10 (80%) were positive with AGPT, while 10/10 (100%) were positive with PCR.

Of the ten tissue specimens from water buffaloes, 7/10 (70%) were positive with AGPT, while 10/10 (100%) were positive with PCR. Of the CAM that were inoculated with the prepared tissue specimens, 8/10 (80%) were positive with AGPT, while 10/10 (100%) were positive with PCR. The AGPT failed to detect LSDV antigen in whole milk or pelleted sedimented samples, while PCR detected the nucleic acid of LSDV in five out of ten (50%) of the whole milk samples and in six out of ten (60%) of the pelleted sedimented samples (Table I).

Propagation and titration of lumpy skin disease virus

Table II shows the propagation and titration of LSDV; the virus titres were $10^{1.32}$, $10^{2.41}$ and $10^{2.73}$ after the first, second and third passage respectively.

Table II
Lumpy skin disease virus propagation and titration

Passage no.	Virus titre
1	101.32 ^(a)
2	102.41
3	102.73

a) Median egg infective dose (EID₅₀)/0.1 ml

Discussion

Cattle are the natural hosts of LSDV; both zebu and exotic breeds are susceptible (27). The present study indicated that the water buffalo is susceptible to natural infection under field conditions with the Neethling poxvirus, the aetiological agent of LSD. Lumpy skin disease virus infection was confirmed in cattle and water buffaloes on the farm of the Faculty of Veterinary Medicine in Kalubia province, and this indicates that LSDV circulates enzootically among cattle and water buffaloes in Egypt.

It has been concluded that wildlife probably does not play a very important role in the perpetuation and spread of LSDV in Africa (20). The present study revealed that water buffaloes are involved in the maintenance cycle of LSD in Egypt and this suggests that LSD has become one of the most important enzootic diseases in the country. The appearance of the disease at the beginning of the summer season may be due to a high prevalence of insect vectors during this period. As a result of the unregulated breeding

Table I

Detection of lumpy skin disease virus in samples from cattle and buffaloes and in inoculated chorio-allantoic membrane by agar gel precipitation test and polymerase chain reaction

Samples	Form used	Agar gel precipitation test		Polymerase chain reaction	
		No. positive/tested	Percentage positive	No. positive/tested	Percentage positive
Tissue samples from cattle*	PS	8/10	80%	10/10	100%
	CAM	8/10	80%	10/10	100%
Tissue samples from water buffaloes*	PS	7/10	70%	10/10	100%
	CAM	8/10	80%	10/10	100%
Milk samples from water buffaloes					
Whole milk	PS	0/10	0%	5/10	50%
	CAM	0/10	0%	0/10	0%
Pelleted sedimented samples	PS	0/10	0%	6/10	60%
	CAM	0/10	0%	0/10	0%
Tank whole milk samples	PS	ND	–	2/10	20%

* Skin nodules

PS: directly prepared sample

CAM: inoculated chorio-allantoic membrane

ND: not done

system and deficient animal husbandry on farms in Egypt, animals are readily exposed to insect vectors that facilitate the transmission of LSDV. Housing the cattle and water buffaloes together on the farm investigated may have helped the spread of the disease among the animals.

In the current study, typical clinical signs of LSD were observed: affected cattle and water buffaloes showed signs of pyrexia and anorexia, followed by the sudden appearance of firm skin nodules, in some cases all over the body. A sharp decrease in milk production and enlargement of the superficial lymph nodes, as well as gradual emaciation of the diseased cattle and buffaloes, were common clinical signs. These signs are similar to those reported previously in cattle (1, 3, 10).

In this study, a large variation in the clinical signs was observed among diseased animals, ranging from mild infection to acute disease with severe clinical manifestations. In general, the number of skin nodules per animal was greater in cattle than in water buffaloes. Given that the water buffalo represents a major proportion of the animal wealth in Egypt, LSDV infection will be of great economic importance owing to the marked reduction in milk production and destruction of the hide.

In both Egypt and Sudan, it has been noticed that LSD is more severe among cattle of foreign breeds, such as Friesian cattle, while it is generally milder in indigenous cattle (1, 24). This study, found that the severity of the clinical signs was greater in cattle than in water buffaloes, and they suffered from larger numbers of skin nodules. This suggests that water buffaloes may be less susceptible to LSD than cattle.

In the current study, the DNA of LSDV was detected using PCR on prepared tissue samples (skin nodules) and on CAM that had been inoculated with tissue samples prepared from cattle and water buffaloes, as well as on whole milk and pelleted sedimented milk samples from water buffaloes. The PCR is a fast and sensitive method of demonstrating LSDV DNA in blood and skin samples from experimentally infected cattle (37). The results of the present study indicate that PCR is more sensitive than AGPT in the diagnosis of LSD.

The harvested CAMs that were positive by AGPT were collected, pooled and prepared, then inoculated onto CAM for further viral propagation and titration. The titre became $10^{2.73}$ median egg infective doses (EID)₅₀/0.1 ml at the third passage. The low titre of LSDV may be attributed to the fact that poxviruses show little receptor-mediated binding to cells, as reported by Doms *et al.* (15), Fearon and Wong (17) and Payne and Norrby (32), therefore it is preferable to isolate and propagate poxviruses in tissue culture rather than in CAM (11, 16, 26, 36).

During this study, AGPT failed to detect LSDV antigen in both whole milk and pelleted sedimented samples, while PCR could detect the nucleic acid of LSDV in five out of ten (50%) of the whole milk samples and in six out of ten (60%) of the pelleted sedimented milk samples from water buffaloes. This suggests that the sensitivity of PCR enabled it to detect the low level of LSDV excreted in the milk of the diseased buffaloes. Chorio-allantoic membranes that were inoculated with the prepared milk samples were negative with both AGPT and PCR. This may be due to the low level of LSDV inoculated into the CAM from the milk samples. Previously, PCR has been used for the detection of LSDV in the semen of experimentally infected bulls (23), for detection of *Capripoxvirus* in nasal, conjunctival and oral secretions of experimentally infected sheep and goats (5), and for detection of SGPV in buffy coats, nasal swabs, oral swabs, scabs and skin lesions as well as in lung and lymph nodes collected at post-mortem examination (6). The present study confirmed the excretion of LSDV in the milk of infected water buffaloes, as detected by PCR.

Lumpy skin disease is of economic importance in enzootic areas. Effective control of LSD requires accurate and rapid laboratory techniques to confirm a tentative clinical diagnosis (37). The current study indicated that PCR is a rapid and sensitive laboratory method for detection of the DNA of LSDV. Rapid diagnosis of LSD outbreaks by PCR will help in the early management of an outbreak. Timely and accurate diagnosis of LSD outbreaks by PCR techniques will also facilitate rapid application of control measures in enzootic areas. Diagnosis of LSD by PCR will therefore play an important role in preventing the spread of the disease.

Conclusion

The present study leads us to conclude that:

- LSD is an important enzootic disease in Egypt, and LSDV is circulating among cattle and water buffaloes in the country
- Egyptian water buffaloes are susceptible to LSDV infection under field and natural conditions
- diseased buffaloes suffer from typical but less severe clinical signs of the disease
- PCR is a rapid, simple, sensitive and accurate method for the detection of LSDV DNA in both clinical specimens, such as skin nodules and milk samples, and in the CAM of inoculated chicken embryos
- rapid diagnosis of LSD outbreaks by PCR will facilitate the rapid application of control measures in enzootic areas
- in developing countries, rapid diagnosis through detection of the nucleic acid of LSDV by PCR is a very good

method of preventing the spread of the disease by insect vectors because it allows rapid removal of positive cases.

– in addition, all live cattle and water buffaloes and their by-products which are imported into Egypt should be tested and determined to be free from LSDV.

Recommendations

The current study leads to the recommendations that:

- mass vaccination should be applied for all cattle and water buffaloes in Egypt, using an effective specific vaccine against LSD, such as the attenuated Neethling strain vaccine or one of the recently developed recombinant vaccines
- fly repellents should be sprayed on cattle and water buffaloes to prevent insects from biting

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L'utilité de l'amplification en chaîne par polymérase pour le diagnostic de la dermatose nodulaire contagieuse chez les bovins et les buffles d'eau en Égypte

S.S.A. Sharawi & I.H.A. Abd El-Rahim

Résumé

En 2006, un foyer de dermatose nodulaire a été enregistré en Égypte chez les bovins et les buffles d'eau. Il a été procédé à une comparaison des résultats obtenus respectivement par l'amplification en chaîne par polymérase (PCR) et par la réaction de précipitation en gélose pour détecter la maladie. La réaction de précipitation en gélose a donné des résultats positifs pour huit des dix échantillons tissulaires prélevés sur des bovins atteints (soit 80 %) ; la PCR a donné des résultats positifs pour les dix échantillons (soit 100 %). Sept des dix échantillons tissulaires prélevés sur des buffles d'eau atteints (soit 70 %) se sont avérés positifs à la réaction de précipitation en gélose ; la PCR a donné des résultats positifs pour les dix échantillons (100 %). Des échantillons de lait de bufflonnes atteintes ont également été analysés ; l'acide nucléique du virus de la dermatose nodulaire contagieuse a été détecté par PCR dans 50 % de ces échantillons ; néanmoins, l'antigène viral n'a pas pu être détecté par la réaction de précipitation en gélose. Les buffles d'eau sont sensibles au virus de la dermatose nodulaire contagieuse. Cette espèce présente des signes cliniques de moindre gravité, mais le virus de la dermatose nodulaire contagieuse est excrété dans le lait. Le recours à la PCR pour détecter les foyers de dermatose nodulaire contagieuse facilitera la mise en œuvre rapide des mesures de prophylaxie. Les auteurs préconisent de vacciner systématiquement les bovins et les buffles d'eau en Égypte, en utilisant un vaccin spécifique contre la dermatose nodulaire contagieuse, par exemple la souche atténuée Neethling, ou un vaccin recombinant.

Mots-clés

Amplification en chaîne par polymérase – Bovin – Buffle d'eau – Dermatose nodulaire contagieuse – Échantillon de lait – Égypte – Épreuve de précipitation en gélose – Foyer – Membrane chorio-allantoïque – Nodule cutané.

La utilidad de la reacción en cadena de la polimerasa para diagnosticar la dermatosis nodular contagiosa en ganado vacuno y búfalos de agua en Egipto

S.S.A. Sharawi & I.H.A. Abd El-Rahim

Resumen

En 2006 se declaró en Egipto un brote de dermatosis nodular contagiosa que afectó al ganado vacuno y a búfalos de agua. Se compararon dos técnicas de detección de la enfermedad: la reacción en cadena de la polimerasa (PCR) y la precipitación en gel de agar (PGA). Con la segunda resultaron positivas ocho de cada diez (80%) muestras tisulares de bovinos enfermos, y con la primera este porcentaje resultó del 100%. Por otro lado, la PGA arrojó resultado positivo en siete de las diez muestras tisulares de búfalos enfermos, y la PCR en todas ellas (un 100%). Previa obtención de diez muestras de leche de búfalas enfermas, la aplicación de la PCR permitió detectar el ácido nucleico del virus de la dermatosis nodular contagiosa en un 50% de ellas, mientras que con la PGA no fue posible detectar el antígeno. Los búfalos de agua son susceptibles a la infección por el virus. Aunque esta especie presentaba signos clínicos de menor gravedad, se observó excreción del virus en su leche. El recurso a la PCR para diagnosticar brotes de dermatosis nodular contagiosa facilitará la rápida aplicación de medidas de lucha. En Egipto convendría proceder a vacunaciones masivas del ganado vacuno y los búfalos de agua empleando una vacuna específica contra la enfermedad, como la vacuna con la cepa atenuada Neethling o una vacuna recombinante.

Palabras clave

Brote – Búfalo de agua – Dermatosis nodular contagiosa – Egipto – Ganado vacuno – Membrana corioalantoidea – Muestra de leche – Nódulo cutáneo – Prueba de precipitación en gel de agar – Reacción en cadena de la polimerasa.



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