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Studies of molecular detection and histopathological examination of lumpy skin diseased cattle

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Abstract

The present research was undertaken at five mandals of Tirupati district of Andhra Pradesh during the year 2022-23 to study the diagnosis of LSD, 40 skin biopsy and whole blood samples were collected randomly from 582 cattle which exhibited classical signs of LSD. Upon PCR examination 40 skin biopsy were positive with a diagnostic rate of 100 per cent, whereas out of 40 whole blood samples 10 (25%) were positive by PCR. Out of 582 cattle eight cattle were positive for both LSD and Theileriosis based on PCR and Five among 40 skin biopsy samples were undergone for histopathological examination and notified that there was an hyperkeratosis of stratum corneum, edema and vacuolization stratum granulosum, severe hydropic degeneration, hyperchromatosis, mononuclear cell infiltration.

Keywords: Andhra Pradesh, Lumpy skin disease, Theileriosis, Skin biopsy and Polymerase chain reaction

Introduction

The world was plagued by an abundance of disease outbreaks during the past 20 years, particularly those driven on by re-emerging viruses such as the Zika virus, the Nipah virus, the severe acute respiratory syndrome virus 2 (SARS-CoV-2), and monkeypox virus (MPXV) (Mourya *et al.*, 2019) [21]. These have been linked to the spillovers from different animal species brought about by increased contact (with humans), mutations in genes that finally brought about variations, and species-crossing (Chadha *et al.*, 2022) [6]. The lumpy skin disease (LSD) is experiencing an unprecedented pandemic in various Asian nations. The lumpy skin disease virus (LSDV) is responsible for this highly contagious and re-emerging viral infection in domestic cattle and Asian water buffaloes. It is a member of the family Poxviridae, subfamily Chordopoxviridae, and the genus Capripoxvirus (Tuppurainen *et al.*, 2017) [30]. Like other poxviruses, LSDV replicates in an infected cell's cytoplasm, generating unique perinuclear viral factories. The LSD virus is a sizable, brick-shaped particle with dimensions of 262- 273 nm in width and 293-299 nm in length. Similar to other poxviruses, the LSDV genome is double- stranded linear DNA with 25 per cent GC content that is 1,50,000 base pairs (bp) in length and encodes about 156 open reading frames (ORFs). At each end of the linear genome, there is a 2200- 2300 bp inverted terminal repeat sequence. This disease is known as 'Neethling virus disease,' 'knopvelsiekte,' 'pseudo-urticaria,' and 'exanthema nodularis bovis' (Tuppurainen *et al.*, 2017; Khan *et al.*, 2021) [30, 18]. Moreover, LSDV is closely related to the goat poxvirus (GTPV) and sheep poxvirus (SPPV), as it shares high sequence similarity and antigenic relationships with them (Diallo and Viljoen, 2007) [9]. The first reports of the lumpy skin disease in India was at 2019, and later on spread to various states, like Gujarat, Rajasthan, Haryana, Delhi, Tamilnadu, Tripura, Telangana, Odisha, Kerala, West Bengal, Maharashtra, Assam, Madhya Pradesh, Jharkhand, Andhra Pradesh, and Chhattisgarh (Datten *et al.*, 2023; Yadav *et al.*, 2021) [8, 31]. Lumpy skin disease is a transboundary, vector- borne, non-zoonotic disease and become a major hazard to livestock around the world in recent years (Gupta *et al.*, 2020) [20]. Using hematoxylin and eosin staining, histopathology of skin lesions offers a way to identify the intracytoplasmic inclusion bodies of LSD virus-infected cells (Tuppurainen *et al.*, 2011) [29]. The disease can be diagnosed by routinely used serological and molecular tests (OIE, 2017).

Hence, the present study was conducted to diagnose the lumpy skin disease by PCR and histopathological changes occurred in the Skin of affected cattle that were located in and around the Tirupati District of Andhra Pradesh.

Materials and Methods

The present study was carried out at household cattle located in five mandals of Tirupati District, Veterinary Clinical Complex, College of Veterinary Science, Tirupati and SLDL, Tirupati, from December 2022 to September 2023. A total of 582 cattle, which exhibited classical signs of LSD were selected among the 40 skin biopsy and 40 whole blood samples were collected for PCR and five were collected for Histopathological Examination. The LSD virus was available at state level diagnostic laboratory, SLDL, Tirupati was used as a positive DNA control during the present study and the collected whole blood sample was stored at -4 °C or at -20 °C,

whereas tissue samples was stored at -70 °C until further processing. These samples were processed as per standard protocol mentioned by OIE, 2017. Isolation of viral DNA from blood samples, tissue homogenate was done using QIAamp® DNA Mini Kit (QIAGEN). The blood samples and tissue samples collected from suspected cases of LSD were confirmed with conventional PCR targeting genus specific P32 gene, species specific GPCR gene, RPO030 gene and Ankyrin repeat gene according to Binopal *et al.* (2001) [4]; Kumar *et al.* (2021) [19]. The amplification reactions were carried out in 0.2 ml micro centrifuge tubes using thermal cycler.

The extracted DNA from all the collected blood and tissue samples were subjected to conventional PCR to detect the presence of Capri pox virus genome using Capri pox virus genus specific primers targeting the P32 gene. The oligonucleotide primers used for PCR.

Oligonucleotide primers used for genus specific P32 gene

Primer Name	Sequence	Base pair size	Reference
P32 F	5'-TTTCCTGATTTTCTTACTA-3'	192	Binopal <i>et al.</i> (2001) [4]
P32 R	5'-AAATTATATACGTAAATAAC -3'		

Nuclease free water served as negative control in each run and amplification was performed in thermo cycler with the following conditions: initial denaturation at 95 °C for 2 minutes, 35 cycles of denaturation at 95 °C for 40 seconds, annealing at 50 °C for 1 minute, extension at 72 °C for 1

minute final extension at 72 °C for 4 minutes and with hold temperature at 4 °C. The total volume of the reaction mixture was made up to 25 µl with nuclease free water. The tubes were then spun for 10 seconds and PCR was carried out in thermal cycler with cycle conditions for amplification.

Commonly used PCR reaction mixture for amplification of all genes

Component	Volume/reaction (µl)
HIGenoMB Master mix	12.5
20 pm forward primer	0.5
20 pm reverse primer	0.5
Template DNA	2
Nuclease free water	9.5
Total volume	25 µl

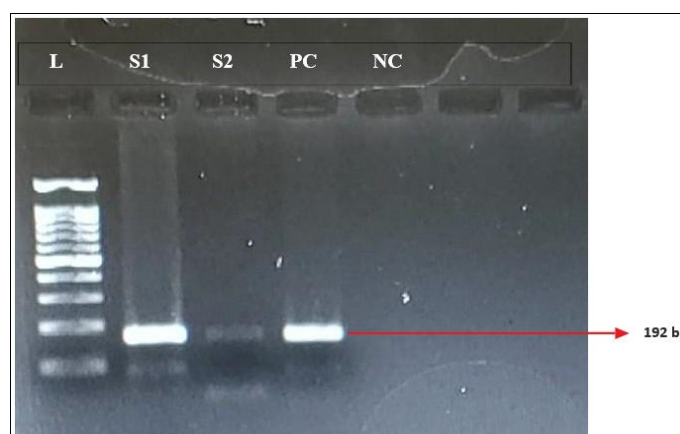
Results

The viral DNA of lumpy skin disease was detected by using polymerase chain reaction using skin biopsy and whole blood samples collected from lumpy skin diseased cattle.

Among 40 skin biopsy and whole blood samples, 40 skin biopsy and 10 whole blood samples were found to be positive for lumpy skin disease and as depicted in Table 01. The details of polymerase chain reaction is depicted in Plate 1

Diagnosis of lumpy skin disease based on polymerase chain reaction

S. No.	Diagnosis	Total number of samples	Total number of positives	Percentage
01	Polymerase chain reaction	Skin biopsy (40)	40	100%
		Whole blood (40)	10	25%



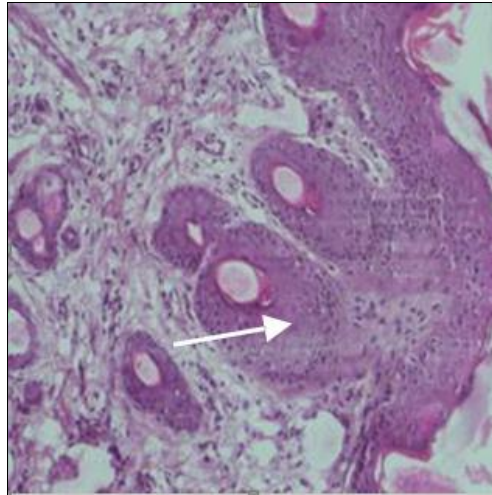
(L-100 bp DNA ladder, S1-S2- Positive samples, PC- Positive control, and NC- Negative control)

Plate 1: Agarose gel showing amplified PCR product of size 192 bp

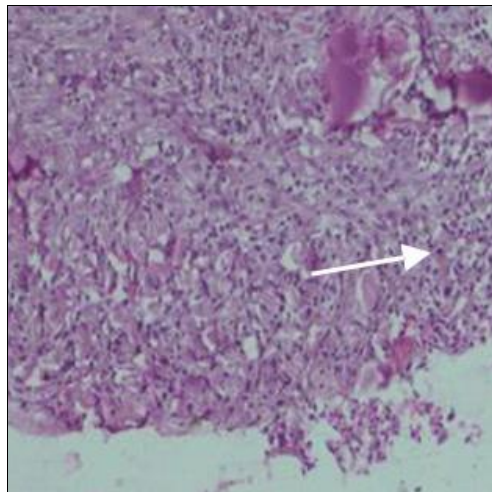
Histopathological examination: From the polymerase chain reaction positive samples, five skin biopsy samples were randomly subjected for histopathological examination. The histopathological findings included, hyperkeratosis of stratum corneum, edema and vacuolization stratum granulosum, severe hydropic degeneration, hyperchromatosis,

mononuclear cell infiltration, follicle of sebaceous gland, fibroblast proliferation, keratin nests formation in the epidermis.

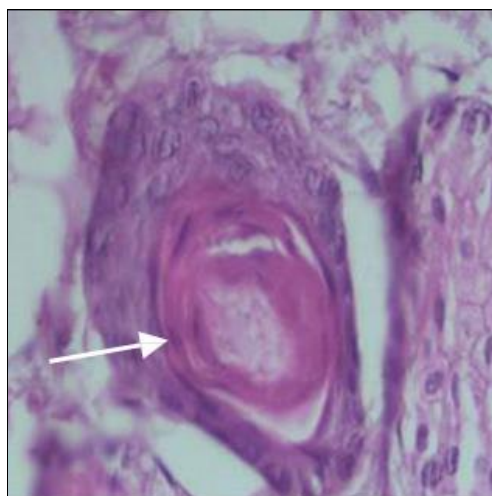
Whereas in dermis mononuclear cell infiltration, presence of intracytoplasmic inclusion bodies, pustule with infiltration of neutrophils, liquefaction necrosis and vasculitis was noticed.



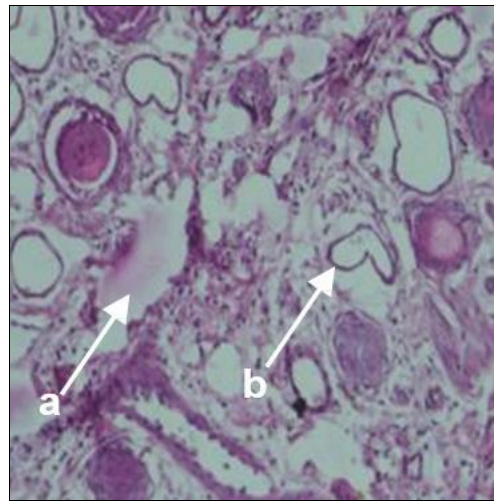
A. Histopathological examination of skin nodule showing hyperkeratosis in the epidermis (arrow). Haematoxylin and Eosin method x160



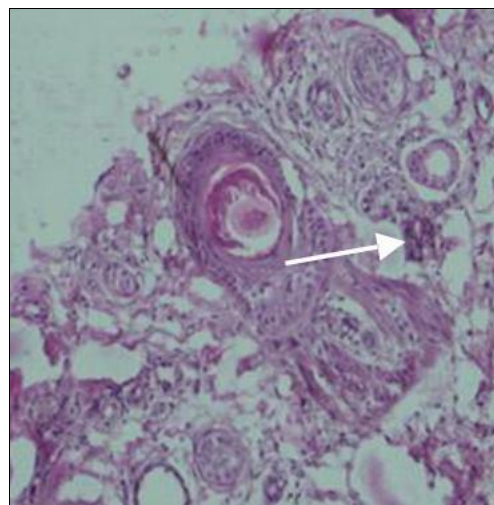
B. Histopathological examination of skin nodule showing Mononuclear cell infiltration (arrow). Haematoxylin and Eosin method x160



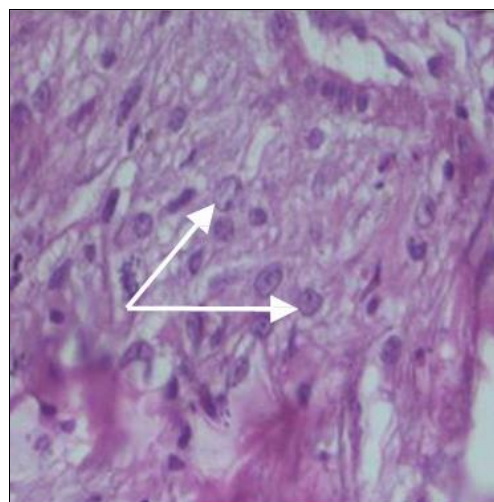
C. Histopathological examination of skin nodule showing fibroblast proliferation and presence of keratin as nest formation (arrow). Haematoxylin and Eosin method x400



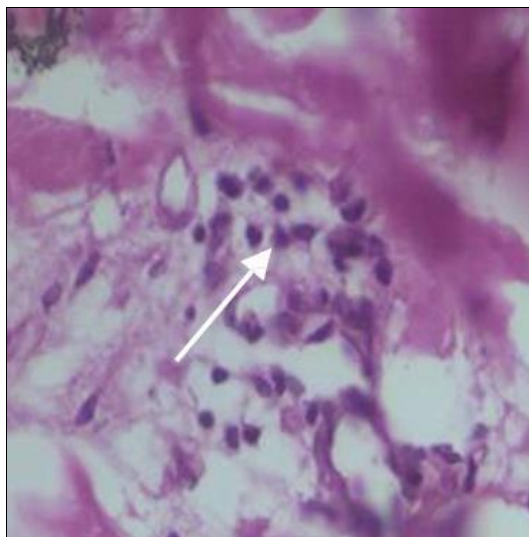
D. Histopathological examination of skin nodule showing (a) Edema (arrow). (b) Vacuolation (arrows). Haematoxylin and Eosin method x160



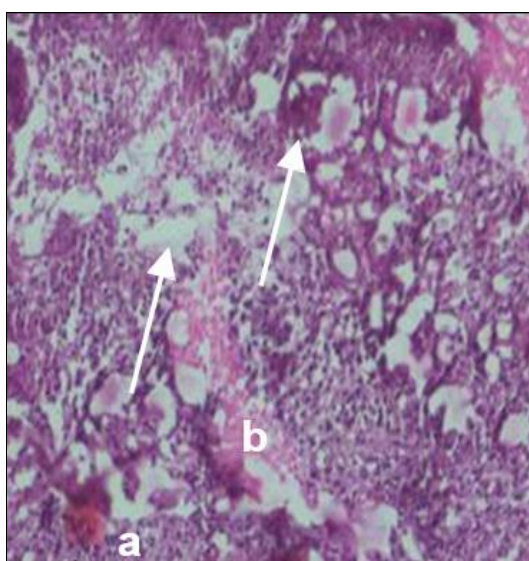
E. Histopathological examination of skin nodule showing pustule with infiltration of neutrophils (arrow). Haematoxylin and Eosin method x160



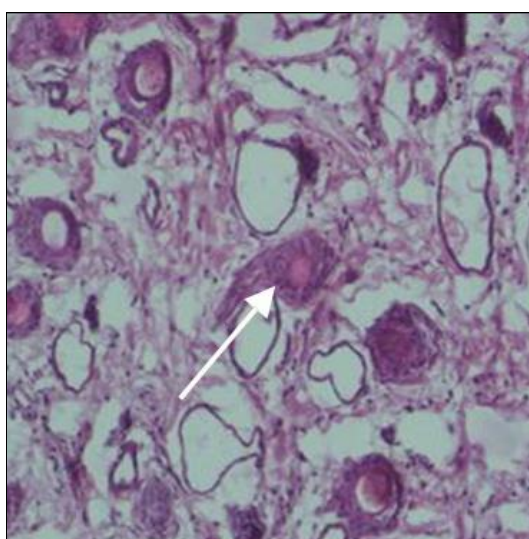
F. Histopathological examination of skin nodule showing intracytoplasmic inclusion bodies with pushed nucleus towards periphery (arrows)



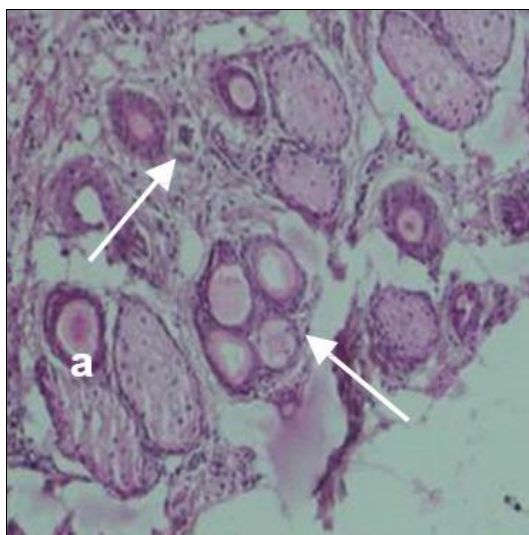
G. Histopathological examination of skin nodule showing hyperchromatosis (arrow). Haematoxylin and Eosin method x160



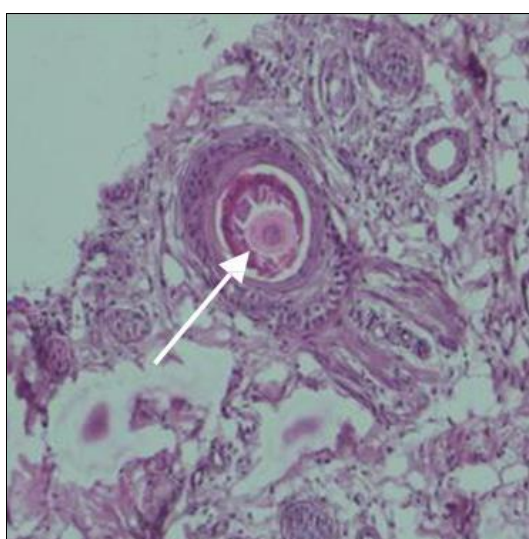
H. Histopathological examination of skin nodule showing (a) liquefaction necrosis (arrow) (b) presence of pus (arrow). Haematoxylin and Eosin method



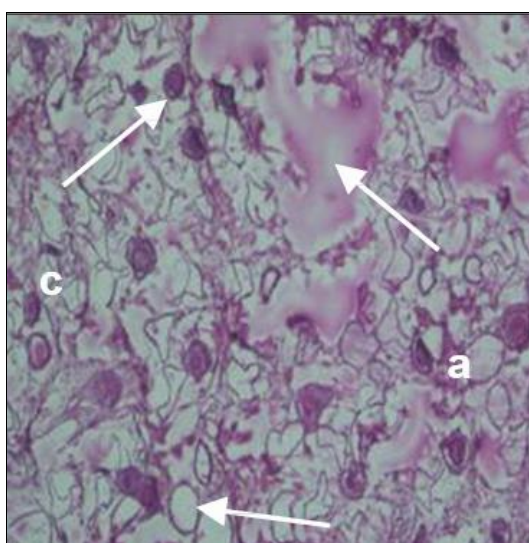
I. Histopathological examination of skin nodule showing follicle of sebaceous gland (arrow). Haematoxylin and Eosin method x160



J. Histopathological examination of skin nodule showing (a) microvessel formation with fibrin depositions (arrow) (b) cell infiltration (arrow). Haematoxylin and Eosin method x160



K. Histopathological examination of skin nodule showing granuloma (arrow). Haematoxylin and Eosin method x160



L. Histopathological examination of skin nodule showing (a). Edema (b). Vacuoles (c) microvessel formation Haematoxylin and Eosin method x160

Discussion

In the present study, primarily lumpy skin disease was diagnosed by clinical signs such as pyrexia, formation of characteristic nodules over the surface of the body, anorexia,

enlargement of lymph nodes, edema of limbs, brisket and ventral abdomen, nasal and ocular discharges and it is in accordance with Mulatu and Feyisa (2018)^[20]; Awad *et al.* (2010)^[3] and Constable *et al.* (2017)^[7]. However, the lumpy

skin disease was confused with other diseases which cause similar skin lesions such as insect bites, demodectosis, allergic skin lesions, anaphylactic reactions, pseudo lumpy skin disease and dermatophilosis (Abdulqa *et al.*, 2016; Constable *et al.*, 2017 and Tuppurainen *et al.*, 2017) [30, 7]. In the present study, laboratory confirmation of lumpy skin disease virus was done by polymerase chain reaction by using skin nodular biopsy samples and whole blood samples. Histopathological confirmation was done with skin biopsy samples and it is in agreement with Naby *et al.* (2021) [22] and Hasib *et al.* (2021) [17].

In the present study, from 40 suspected cattle, whole blood and skin biopsy samples were subjected for polymerase chain reaction. Out of them 10 (25%) whole blood samples which were collected from pyrexia cases and 40 (100%) skin biopsy samples which were positive on polymerase chain reaction. The present findings were in agreement with Awad *et al.* (2010) [3]. Similarly, Prabhu (2022) [27] found less positivity (2/23) in whole blood samples tested by polymerase chain reaction.

In the initial stage of infection, viremic phase lasts for about three to five days, then the virus is disseminated to subcutaneous and intradermal tissues in later stage of infection (Mulatu and Feyisa, 2018) [20] which might be the reason for less positivity with whole blood. However, 41.02 per cent (32/78) positivity with whole blood samples was recorded by Elhaig *et al.* (2017) [11].

In our study, the positivity for skin biopsy samples through polymerase chain reaction was 100 per cent, similarly 90.14 per cent positivity from skin biopsy samples was detected by polymerase chain reaction by targeting P32 gene (Gharban *et al.*, 2019) [14] and 89.7 per cent positivity was recorded earlier by Elhaig *et al.* (2017) [11]. The higher per cent positivity with polymerase chain reaction in tissue biopsy might be due to its capability to detect even low genomic copies of the lumpy skin disease virus (Al-Salihi, 2014 and Awad *et al.*, 2010) [2, 3]. Further, it was documented that the lumpy skin disease virus persists in the nodular tissue for more than 120 days (Gumbe, 2018) [15], facilitating for greater positivity over longer period of time.

The polymerase chain reaction is considered as a confirmatory test, quick and easy to interpretate the results, also it can detect the viral antigen even in the dead state, which was in agreement with (Al-Salihi, 2014; Awad *et al.*, 2010) [2, 3].

Other sample of choice for polymerase chain reaction can be nasal swabs, oral swabs, saliva, lacrimal secretions, milk and semen as the virus is proved to be excreted in these samples (Farag *et al.*, 2020) [12].

The histopathological changes noticed in the skin biopsy samples of the present study included, hyperkeratosis of stratum corneum, edema and vacuolization stratum granulosum, severe hydropic degeneration, hyperchromatosis, and mononuclear cell infiltration. This is in agreement with earlier reports by Salib and Osman (2011) [28]; Gharban *et al.* (2019) [14]. Follicle of sebaceous gland, fibroblast proliferation, keratin nests formation was noticed in the epidermis. Whereas, in dermis mononuclear cell infiltration, presence of intracytoplasmic inclusion bodies, pustule with infiltration of neutrophils, liquefaction necrosis and vasculitis were observed and it is corroborated with the findings of Gharban *et al.* (2019) [14]. The confirmation of lumpy skin disease was done by histopathology, based on the presence of

eosinophilic intracytoplasmic inclusion bodies in particular in the prickle cell layer.

Histopathology was considered as a precised diagnostic method which provides a useful information about a tissue and its architecture in the diagnosis of lumpy skin disease (Otranto *et al.*, 2004) [26].

The World Organization for Animal Health (OIE) recommends that the following methods be used to identify antibodies against lumpy skin disease virus such as virus neutralization test, agar gel immunodiffusion test, indirect fluorescence test, ELISA, and western blot (OIE, 2016). The virus neutralization test is regarded as "the gold standard" and the most precise way to find antibodies against LSDV, its sensitivity is insufficient to determine the identity of every animal that came into contact with the virus (EFSA, 2015). While, western blot is sensitive and specific enough for routine use, it is difficult to set up and expensive. Standardization of the coated antigen in the microtiter plates is necessary for ELISA. Less specific techniques for detecting antibodies against LSDV include immunodiffusion and immunofluorescence (Bowden *et al.*, 2009 and OIE, 2016) [5, 24]. Conversely, the primary benefits of ELISA include its affordability, rapidity, and ability to test a large number of samples on a single plate (Gari *et al.*, 2008) [13]. Using hematoxylin and eosin staining, histopathology of skin lesions offers a way to identify the intracytoplasmic inclusion bodies of LSD virus-infected cells (Tuppurainen *et al.*, 2011) [29].

Conclusion

In the present study, notably, a positivity rate of 100 per cent was observed in nodular skin biopsy samples, while whole blood samples exhibited 25 per cent positivity and histopathology may show the changes occurred in the skin of the affected cattle.

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