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H Jaferin
Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

RP Aravindh Babu
Translational Research Platform for Veterinary Biologicals, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

TMA Senthilkumar
Zoonoses Research Laboratory, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

G Dhinakar Raj
Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

R Sridhar
Central University Laboratory, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

A Ramesh
Vaccine Research Centre Viral Vaccine, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

Corresponding Author
TMA Senthilkumar
Zoonoses Research Laboratory, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

Molecular detection of lumpy skin disease virus from clinically infected cattle of Tamil Nadu

H Jaferin, RP Aravindh Babu, TMA Senthilkumar, G Dhinakar Raj, R Sridhar and A Ramesh

Abstract
Lumpy skin disease (LSD) in cattle population continues to be a major economic threat among dairy farmers worldwide. Accurate diagnosis of LSD in animals is therefore of utmost importance to reduce the financial burden of farmers. Although there are several methods to detect LSDV in clinical samples, PCR remains the most sought-after method due to its relatively short assay time. In this pilot study we have established a diagnostic PCR method to amplify the complete A33R gene of LSDV genome. This new method is comparable with that of the PCR method prescribed by OIE for diagnosis of LSDV in clinical samples. Furthermore, the complete coding region of the A33R-gene from one of the field isolates was sequenced.

Keywords: LSDV, diagnosis, PCR, A33R primers

1. Introduction
Lumpy skin disease (LSD) is widespread throughout most of Africa and parts of the Middle East and it was exotic to India until 2019. The etiological agent Lumpy skin disease virus (LSDV) is a double-stranded DNA virus belonging to the genus Capripoxvirus. It is genetically and antigenically related to the sheep pox (SPPV) and goat pox (GTPV) viruses (Weiss, 1968) [14]. Cattle, water buffalo and wild ruminants are all susceptible to lumpy skin disease. Cattle of all ages and breeds are affected, although young cattle and those towards the end of their lactation are particularly vulnerable (Tuppurainen and Qura, 2012) [13]. Sheep and goats appear to be immune to the virus (Shen *et al.*, 2011) [10].
LSD is characterized by fever and skin nodules throughout the body. Besides skin, lesions also extend to subcutaneous tissues, muscles and internal organs (Weiss, 1968, Tageldin *et al.*, 2014) [14, 11]. Lumpy skin disease has also caused significant economic losses including reduction in milk yield, damaged hides, decreased growth rate in beef cattle, sterility, abortion and eventually death of severely affected animals (Abutarbush *et al.*, 2015, EFSA, 2015) [1, 6].
The disease is mechanically transmitted by blood feeding arthropods (Carn, 1995) [3]. The prevalence of insect vectors and host susceptibility both play a significant role in the morbidity and mortality rate of LSD. Mortality rates in endemic areas range from 1% to 3%, while morbidity is often about 10% (Davies, 1991, Coetzer, 2004 and Babiuk *et al.*, 2008) [5, 4, 2].
The tentative diagnosis is based on distinctive clinical symptoms. Lab based methods of diagnosis include, virus isolation, polymerase chain reaction, electron microscopy, virus neutralization and indirect fluorescent antibody technique (IFAT) (OIE, 2021) [9]. Conventional gel-based PCR prescribed by OIE is often the test of choice (Tuppurainen *et al.*, 2005) [12], however OIE prescribed method yields only a 192 bp which is often unsuitable for phylogenetic analysis. In this study we have established a PCR method for LSDV diagnosis based on an immunodominant LSDV A33R gene. This PCR followed by sequencing facilitates phylogenetic analysis of the field isolates.

2. Materials and Methods
2.1. Samples collection
Skin scab and blood samples used in this study were collected from cattle suspected to have LSD and submitted to Central University Laboratory, Madhavaram, Chennai during the year 2020.The details of the test samples are summarised in Table.1. DNA extracted from Vero cell passaged LSDV isolate maintained at Translational Research Platform for Veterinary Biologicals laboratory (TRPVB), TANUVAS was used as positive control.

Table 1: Comparison of A33R-based PCR assay and p32-based PCR assay for the detection of LSDV in skin scab samples and blood samples of cattle from different places of Tamil Nadu suspected for LSD

Sl. No.	Sample ID	Host	Kind of Sample	Place of origin	p32 primers	A33R primers
1	LSDV9587	Cattle	Skin scrapings	ADIU, Tiruvannamalai	✓	✓
2	LSDV9744	Cattle	Skin scrapings	ADIU, Tiruvallur	✓	✓
3	LSDV10296	Cattle	Skin scrapings	ADIU, Erode	x	x
4	LSDV10297	Cattle	Skin scrapings	ADIU, Erode	x	x
5	LSDV10455	Cattle	Skin scrapings	ADIU, Ramanathapuram	✓	✓
6	LSDV8488	Cattle	Blood sample	ADIU, Salem	x	x
7	LSDV754	Cattle	Skin scrapings	ADIU, Erode	x	x
8	LSDV11530	Cattle	Skin scrapings	ADIU, Tiruvallur	✓	✓
9	LSDV9187	Cattle	Blood sample	VUTRC, Rajapalayam	x	x
10	LSDV9201	Cattle	Blood sample	VUTRC, Rajapalayam	x	x
11	LSDV9095	Cattle	Skin scrapings	ADIU, Dindigul	✓	✓
12	LSDV2123	Cattle	Skin scrapings	ADIU, Kanchipuram	✓	✓
13	LSDV2154	Cattle	Blood sample	VCRI, Thanjavur	x	x
14	LSDV2161	Cattle	Blood sample	VCRI, Thanjavur	x	x
15	LSDV2163	Cattle	Blood sample	VCRI, Thanjavur	x	x
16	LSDV9772	Cattle	Skin scrapings	ADIU, Kanchipuram	✓	✓
17	LSDV9776	Cattle	Skin scrapings	ADIU, Kanchipuram	✓	✓
18	LSDV9920	Cattle	Skin scrapings	ADIU, Kanchipuram	✓	✓
19	LSDV2930	Cattle	Blood sample	ADIU, Perambalur	x	x
20	LSDV2931	Cattle	Blood sample	ADIU, Perambalur	x	x
21	LSDV2934	Cattle	Blood sample	ADIU, Perambalur	x	x
22	LSDV10096	Cattle	Skin scrapings	Goshala, Tiruvallur	✓	✓
23	LSDV2922	Cattle	Blood sample	ADIU, Karur	x	x
24	LSDV2695	Cattle	Blood sample	VUTRC, Madurai	x	x
25	LSDV3038	Cattle	Blood sample	VCRI, Namakkal	x	x
26	LSDV3499	Cattle	Blood sample	MVC, Chennai	x	x
27	LSDV3178	Cattle	Skin scrapings	VUTRC, Madurai	✓	✓
28	LSDV3481	Cattle	Blood sample	ADIU, Salem	x	x
29	LSDV3483	Cattle	Blood sample	ADIU, Salem	x	x
30	LSDV3485	Cattle	Blood sample	ADIU, Salem	x	x
31	LSDV3486	Cattle	Blood sample	ADIU, Salem	x	x
32	LSDV326	Cattle	Skin scrapings	ADIU, Madurai	✓	✓
33	LSDV11532	Cattle	Blood sample	ADIU, Tiruvallur	x	x
34	LSDV9086	Cattle	Blood sample	ADIU, Namakkal	x	x
35	LSDV9088	Cattle	Blood sample	ADIU, Namakkal	x	x
36	LSDV10620	Cattle	Blood sample	ADIU, Perambalur	x	x
37	LSDV10625	Cattle	Blood sample	ADIU, Perambalur	x	x
38	LSDV10545	Cattle	Skin scrapings	ADIU, Ramanathapuram	✓	✓
39	LSDV10546	Cattle	Skin scrapings	ADIU, Ramanathapuram	✓	✓
40	LSDV2155	Cattle	Blood sample	VCRI, Thanjavur	x	x

✓ indicates the presence of LSDV genome

x indicates no LSDV genome detected

- ADIU - Animal Disease and Intelligence Unit
- VUTRC - Veterinary University Training and Research Centre
- VCRI - Veterinary College and Research Institute
- MVC - Madras Veterinary College

2.2. Viral DNA extraction

Tissue suspension was made by grinding the samples in pestle and mortar and homogenate was stored at – 80 °C until used. Viral DNA was extracted from scab tissue homogenate supernatant using the High pure viral nucleic acid kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Briefly, 200 µl of sample supernatants were subjected to lysis step at 72 °C for 10 min followed by addition of binding buffer, inhibitor removal buffer and wash buffer. The DNA was then eluted in 30 µl of elution buffer. The DNA concentration was detected by Multimode reader (Tecan Infinite M200 PRO).

2.3. PCR with primers for p32 and A33R genes

All the DNA samples were first tested by gel-based PCR with OIE prescribed p32 primers (Table. 2). Amplification of DNA template was carried out using the Taq 2X PCR master mix RED (Amplicon, Denmark) containing 10 pmol each of forward and reverse primers (Table.1) in a 12.5 µl reaction volume. The amplifications were performed on a Bio-Rad C 1000 Touch Thermocycler. After denaturation at 94 °C for 5 min, the reactions were cycled 35 times at 94 °C for 30 sec, 54 °C for 30 sec, 72 °C for 30 sec. This was followed by a final elongation step at 72 °C for 5 min.

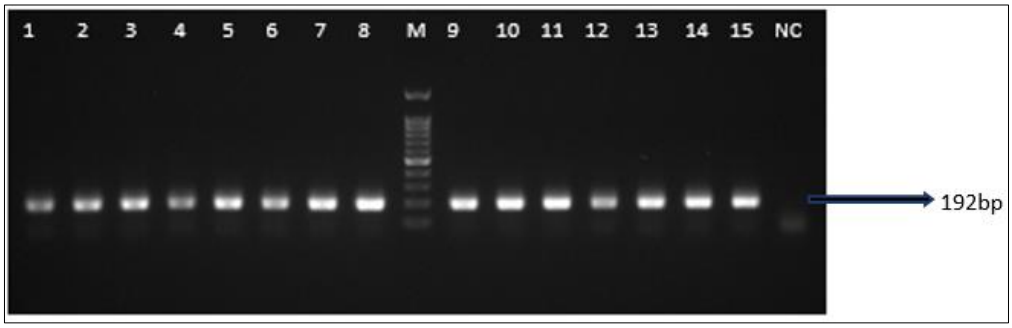
For PCR amplification of A33R gene with A33R primers (Table.2) the thermal cycling conditions were, denaturation at 94 °C for 5 min, the reactions were cycled 35 times at 94 °C for 45 sec, 57.7 °C for 45 sec, 72 °C for 45 sec. This was followed by a final elongation step at 72 °C for 10 min. DNA extracted from Vero cell passaged LSDV isolate maintained at TRPVB laboratory, TANUVAS was used as positive control.

Table 2: List of primers used for PCR

Sl. No.	LSDV Gene	Primer sequence	Amplicon size	Reference
1	p32	F: 5′- TCCGAGCTCTTTCCTGATTTTTCTTACTAT-3′ R: 5′- TATGGTACCTAAATTATATACGTAAATAAC- 3′	192 bp	Ireland & Binepal, 1998; Tuppurainen *et. al*., 2005 [7, 12]
2	A33R	F: 5′-CCGGTCGACATGTTAGTTGATATTCCAAAGAGT -3′ R: 5′-CCGGGATCCTTAAAAAAAAGATCTTACACAGTAATAGC-3′	588bp	Le Goff *et al*., 2009 [8]

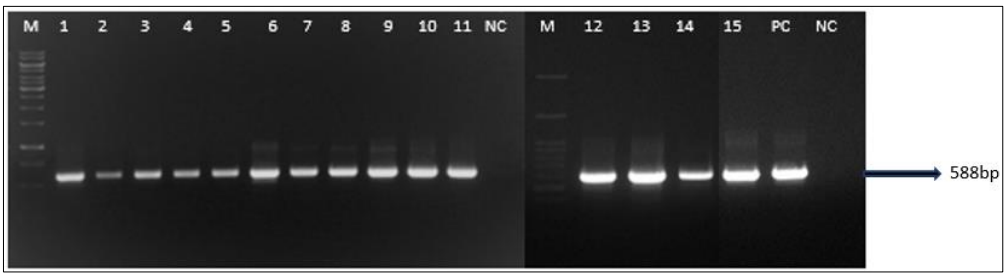
2.4. Sequencing
The A33R gene amplified from one of the isolates was sequenced by Sanger method using commercial sequencing services (Eurofins Genomics India Private Limited. Bangalore). The sequenced data was then assembled with the Seq Man II program of DNASTAR software.

3. Results and Discussion
Out of 40 samples 15 were found positive for LSDV by both the PCRs (Table.1). The p32 PCR amplified a product size of 192 bp (Fig.1). The A33R PCR amplified a product size of 588 bp (Fig.2).



Lane NC: No template control

Fig 1: PCR using p32 primer pair to detect the LSDV virus genome, Lane 1-15: samples positive for LSDV (192 bp), Lane M: DNA ladder



Lane PC: Positive control, Lane NC: No template control

Fig 2: PCR using A33R primer pair to detect the LSDV virus genome, Lane 1–15: samples positive for LSDV (588 bp), Lane M: DNA ladder

Agreement between both the PCRs were measured by calculating the Cohen's kappa coefficient (j). The sensitivity of the A33R based PCR assay was found to be 100% in comparison with p32 based PCR assay. The Cohen's kappa coefficient for the agreement between the two assays is 1.0 indicating a perfect agreement. The A33R gene sequence of one of the isolates was submitted to Gen Bank with Accession No. OP221022. Sequence data obtained from field isolates may aid in identification of any variants. PCR methods for detection of LSDV genome are widely accepted and are useful even in resource constrained regions. Since emergence of LSD in India during 2019, it continues to wreak havoc among the cattle population throughout the country. At the time of writing this manuscript, more than 3000 cattle died in the states of Rajasthan and Gujarat due to LSD.

4. Conclusion
In the present study, we have established a diagnostic PCR method that amplifies the complete A33R gene of LSDV genome. The result of A33R PCR is comparable to that of the p32 gene-based PCR prescribed by the World Organisation for Animal Health (WOAH) for diagnosis of LSDV in clinical samples. Sequence analysis of the immunodominant A33R gene of the field isolates can be useful for better understanding of the LSDV variants and the spread pattern of the outbreak.

Footnote: This work is a part of the Ph.D. thesis work done by the first author.

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