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Comparative evaluation of polymerase chain reaction assays for the detection of lumpy skin disease virus genome

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Abstract
Lumpy skin disease is an economically devastating and transboundary disease in bovine. The disease has been emerging into Asia and Pacific regions in recent times. Polymerase chain reaction (PCR) based molecular detection of Lumpy skin disease virus (LSDV) genome is the most appropriate method in terms of time and cost. The present study compares the analytical sensitivity of various PCR assays targeting the LSDV genome using 5 different gene specific primers. Based on their analytical sensitivity or limit of detection, it is recommended that the P32 PCR is the most sensitive one for detection of LSDV genome in field samples.

Keywords: PCR, Diagnosis, TCID50, Analytical sensitivity, LSD

Introduction
Lumpy skin disease (LSD) caused by Lumpy skin disease virus (LSDV), a double stranded deoxyribonucleic acid virus of the genus Capripoxvirus, family Poxviridae, is categorized as a notifiable disease by the World Organization for Animal Health (WOAH) because of rapid transboundary spread and cause of substantial losses in cattle production and reproduction. It causes decrease in milk and meat production, damaged skins and hides, draught power loss, abortions, fertility problems, as well as death or culling of sick cattle (Molla *et al*., 2017) [9]. It is characterised by fever, lymph adenopathy and nodules, which are randomly distributed and range in diameter from 0.5 to 5 cm, involve both the skin and subcutaneous tissues and sometimes even the underlying musculature (Coetzer and Tuppurainen, 2004) [4].
The disease is endemic across Africa and in the Middle East. Since 2015, the disease has spread into the Balkans, the Caucasus and the southern Russian Federation. More recently, it has been reported in the Asia and Pacific regions including India, China, Nepal, Sri Lanka, Bhutan, Bangladesh and represent a cause of serious concern for the livestock and dairy industries (Azeem *et al*., 2022) [2]. In India, the disease was first reported in 2019 (Sudhakar *et al*., 2020) [10] and within the past 3 years it has become endemic in almost all parts of India.
Accurate, early, rapid and sensitive method of diagnosis of disease is very essential. Nucleic acid detection methods have become the norm that fulfils the requirement of sensitive and specific detection of the pathogen genome. Of the several nucleic acid based detection platforms, the polymerase chain reaction (PCR) has now become a routinely employed test for several pathogens including LSDV (Awad *et al*., 2010) [1]. PCR normally targets the gene, that is conserved across viruses of that genus or otherwise depending on the requirement, abundantly expressed and employ the primer pairs that has the highest analytical sensitivity.
In this study, we have evaluated the analytical sensitivity of PCR detection systems, targeting the viral attachment protein (P32) gene, fusion (F) gene, A33 gene, G-protein-coupled chemokine receptor (GPCR) gene and 30 kDa DNA-dependent RNA polymerase subunit (RPO30) gene of capripoxviruses to identify the appropriate target gene to be recommended and employed for LSDV genome detection in routine diagnosis.

Materials and methods Samples
LSDV was isolated and titrated on Madin-Darby Bovine Kidney (MDBK) cell line available at the cell culture lab with ISO class 7 classifications and BSL-2 facility of the Translational Research Platform for veterinary Biologicals (TRPVB), TANUVAS. The infectious titre of the viral stock used in the study, performed by Reed and Muench method on MDBK cells, was104 TCID50/mL.

DNA extraction

DNA was extracted by QIAamp® DNA Mini Kit (Qiagen, Netherland) according to the manufacturer's instruction from theserial dilutions of 104.0 to 0.001 TCID50 per mL of LSDV and is usedfor the establishment of analytical sensitivity of PCR assays.

Polymerase chain reaction

The isolated DNA was subjected to conventional gel based PCR detection systems, targeting the P32 gene, F gene, A33 gene, GPCR gene and RPO30 gene of capripoxviruses using the primers described previously. The details of the primer sequence are mentioned in Table 1.

The PCR amplification was carried out in 12.5 µL reaction mixture containing 2x Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark), 0.4 µM each of the forward and reverse primers and 2 µL of sample DNA. The thermocycling conditions for P32 gene were as follows: initial denaturation at 95 ºC for 5 min, followed by 35 cycles of denaturation at 95 ºC for 30 secs, annealing at 54 ºC for 30 secs, extension at 72 ºC for 30 secs with a step of final extension at 72 ºC for 5 min. The thermocycler conditions for other geneswere as follows: initial denaturation at 95 ºC for 5 min, followed by 35 cycles of denaturation at 95 ºC for 30 secs, annealing at 52.5 ºC for 40 secs for both GPCR and RPO30 genes, 64.5 ºC for 40 secs for A33, 60ºC for 40 secs for F gene and extension at 72 ºC for 1 min with a step of final extension at 72 ºC for 5 min.

The amplified PCR products were subjected to electrophoresis in 1.5% Tris acetate EDTA agarose gel (Seakem LE agarose, USA) along with 100 bp reference molecular weight marker (Genedirex) and visualized under gel doc XR+ gel documentation system (Bio-Rad, USA).

The analytical sensitivity was calculated as the lowest dilution of the DNA in terms of infective virus titre that gives a visible band on agarose gel electrophoresis after PCR amplification.

Table 1: Details of gene-specific primers used for the detection of LSDV genome.

S. No.	LSDV Gene	Sequence	Amplicon size	Reference
1.	P32	F: 5′- TCCGAGCTCTTTCCTGATTTTTCTTACTAT-3′ R: 5′- TATGGTACCTAAATTATATACGTAAATAAC- 3′	192 bp	Ireland and Binepal, 1998 [6]
2.	F	F: 5′- ACTAGTGGATCCATGGACAGAGCTTTATCA-3′ R:5 ′-GCTGCAGGAATTCTCATAGTGTTGTACTTCG-3′	472 bp	Ireland and Binepal, 1998 [6]
3.	A33	F: 5′-CCGGTCGACATGTTAGTTGATATTCCAAAGAGT -3′ R: 5′-CGGGATCCTTAAAAAAAAGATCTTACACAGTAATAGC-3′	584 bp	Jaferin *et al.*, 2022 [5]
4.	GPCR	F: 5′-TTAAGTAAAGCATAACTCCAACAAAAATG-3′ R: 5′-TTTTTTTATTTTTTATCCAATGCTAATACT-3′	1184 bp	Le Goff *et al.*, 2009 [7]
5.	RPO30	F: 5′-CCCCCACATATCAAGACATAGAA-3′ R: 5′-CCATTTAGGTGGATTCGATCTT-3′	821 bp	Jaferin *et al.*, 2022 [5] (unpublished)

Results and Discussion

LSDV containing 104 TCID50 was serially diluted to contain tenfold lowering dilutions of virus even into sub genomic levels. DNA was extracted from each dilution and used as a template for PCR with 5 different gene targets. The analytical sensitivity of PCR was assessed in terms of the minimum TCID50 per mL from which the extracted DNA gave a positive amplification in PCR.

The analytical sensitivity of each target is shown as specific amplified product size of 192 bp (P32, Figure 1), 472bp (F, Figure 2), 584 bp (A33, Figure 3), 1184 bp (GPCR, Figure 4), 821 bp (RPO30, Figure 5) with respective primers.

The analytical sensitivity of PCR targeting P32, F, A33, GPCR and RPO30 genes of LSDV was found to be 0.1, 1, 10, 1 and 1 TCID 50 per mL respectively (Figure 1 to 5).

The sensitivity of detection of pathogen genome depends of the abundance of the target; primer binding efficiency to the chosen gene target, the thermal cycling conditions, the type of enzymes used and so on. In our study the PCR primers of P32 gene had the greatest sensitivity of 0.1 TCID50 and the A33 gene has the lowest sensitivity of 10 TCID50. The PCR for P32 gene therefore is 100 times more sensitive than A33 PCR. When the P32 primers are used in the diagnosis of LSDV from field samples, this is likely to pick the most number of samples positive. When the infective virus in the target sample is between 0.1 and 10 TCID 50 this may be positive only by P32 PCR and not by A33 PCR.

PCR for the detection of LSDV genome have targeted the genes P32, F (Ireland and Binepal, 1998) [6], GPCR (Le Goff *et al*., 2009) [7] and RPO30 (Lamien *et al*., 2011) [10]. However, they have not mentioned the analytical sensitivity in their studies except for P32. The results of present study are in accordance with previous report (Ireland and Binepal, 1998) [6], which states that TCID50 of virus can be detected by PCR targeting P32 gene of LSDV, whereas the ELISA requires 101.5 TCID50 to give a positive result (Carn, 1995) [3].

OIE has recommended the P32 gene specific primers for diagnosis of LSD, which was found to be the most sensitive primers in this study. Hence, it is recommended that P32 gene specific primers be ideally used for PCR based diagnosis of LSD by virtue of its highest analytical sensitivity.

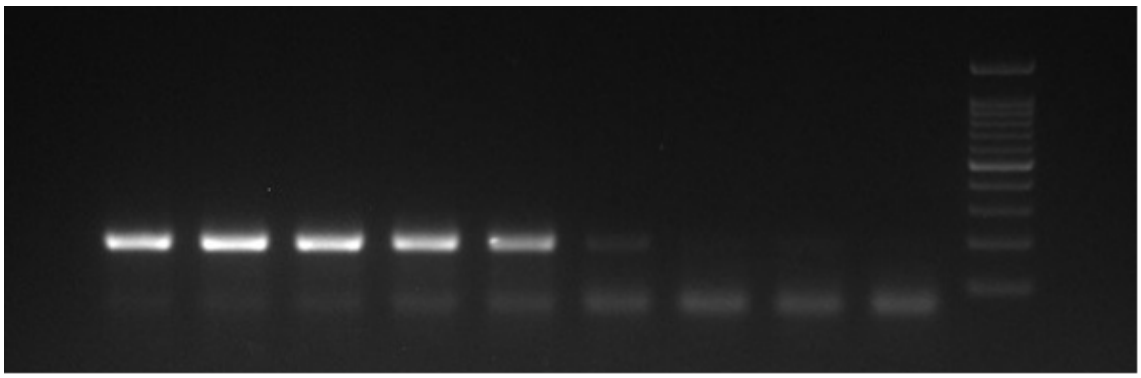

Figure 1: Analytical sensitivity of PCR targeting P32 gene of *LSDV* genome
Agarose gel electrophoresis showing the result of analytical sensitivity of conventional PCR based amplification of 192 bp product, using P32 gene-specific primers. Lane 1-8: PCR performed with DNA extracted (QIAamp method) from 10^4, 10^3, 10^2, 10^1, 1, 0.1, 0.01, 0.001 TCID 50 per mL of LSDV cultured on MDBK cell line, Lane 7: No template control and Lane 10: 100 bp ladder.

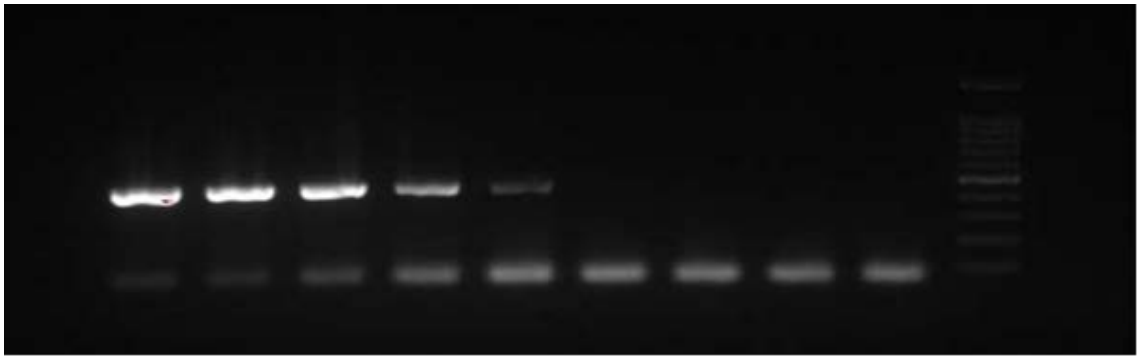

Figure 2: Analytical sensitivity of PCR targeting F gene of *LSDV* genome
Agarose gel electrophoresis showing the result of analytical sensitivity of conventional PCR based amplification of 472 bp product, using F gene-specific primers. Lane 1-8: PCR performed with DNA extracted (QIAamp method) from 10^4, 10^3, 10^2, 10^1, 1, 0.1, 0.01, 0.001 TCID 50 per mL of LSDV cultured on MDBK cell line, Lane 7: No template control and Lane 10: 100 bp ladder.

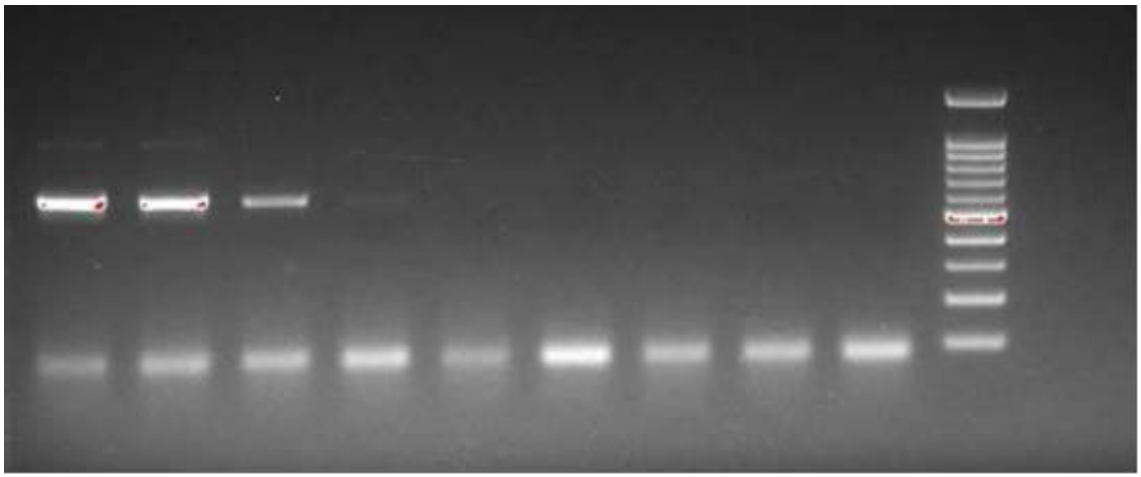

Figure 3: Analytical sensitivity of PCR targeting A33 gene of *LSDV* genome
Agarose gel electrophoresis showing the result of analytical sensitivity of conventional PCR based amplification of 584 bp product, using A33 gene-specific primers. Lane 1-8: PCR performed with DNA extracted (QIAamp method) from 10^4, 10^3, 10^2, 10^1, 1, 0.1, 0.01, 0.001 TCID 50 per mL of LSDV cultured on MDBK cell line, Lane 7: No template control and Lane 10: 100 bp ladder.

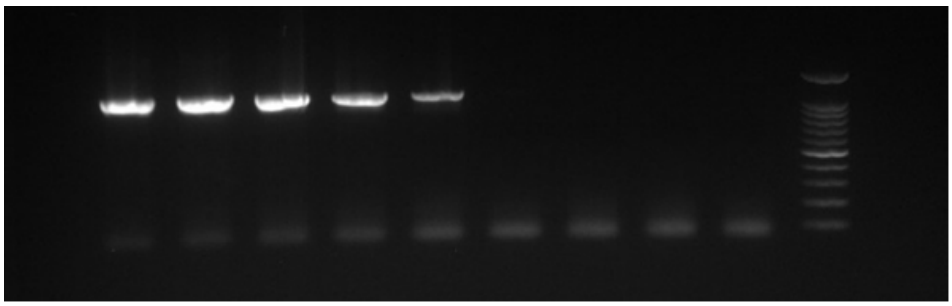

Figure 4: Analytical sensitivity of PCR targeting GPCR gene of *LSDV* genome
Agarose gel electrophoresis showing the result of analytical sensitivity of conventional PCR based amplification of 1184 bp product, using GPCR gene-specific primers. Lane 1-8: PCR performed with DNA extracted (QIAamp method) from 10^4, 10^3, 10^2, 10^1, 1, 0.1, 0.01, 0.001 TCID 50 per mL of LSDV cultured on MDBK cell line, Lane 7: No template control and Lane 10: 100 bp ladder.

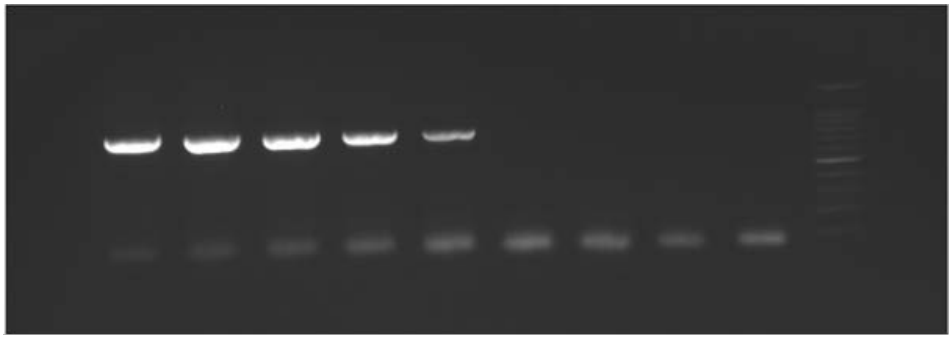

Fig 5: Analytical sensitivity of PCR targeting RP030 gene of LSDV genome Agarose gel electrophoresis showing the result of analytical sensitivity of conventional PCR based amplification of 821 bp product, using RP030 gene-specific primers. Lane 1-8: PCR performed with DNA extracted (QIAamp method) from 104, 103, 102, 101, 1, 0.1, 0.01, 0.001 TCID 50 per mL of LSDV cultured on MDBK cell line, Lane 7: No template control and Lane 10: 100 bp ladder.

Conclusion

PCR assay targeting P32 gene of LSDV is the most suitable method of diagnosis due to its highest analytical sensitivity.

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

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