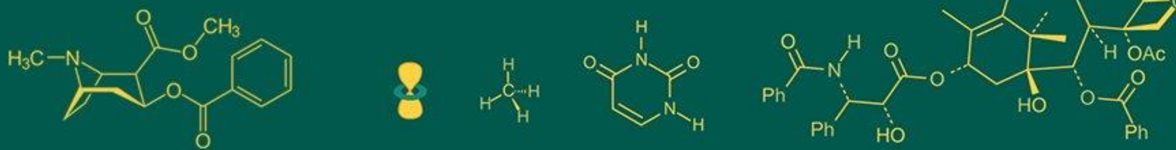

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Polymerase chain reaction based lumpy skin disease diagnostic test using viral p32 gene

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Abstract
Infectious diseases of animals pose a significant threat to animal health and food security. Recently, several viruses have created significant losses in animal life, economy of India and across the globe. Lumpy Skin Disease (LSD), caused by Lumpy skin disease virus (LSDV) has become rampant among cattle in several northern states of India with dire consequences. LSDV is grouped under genus Capripoxvirus of family Poxviridae. Capripoxviruses are cross-reactive within the genus, therefore sheep pox or goat pox vaccines have been used to provide cross protection against lumpy skin disease virus. The diagnosis of LSDV relies on ELISA, FAT, western blotting, Polymerase chain reaction, etc. PCR being the most preferred diagnostic test for LSDV, however the method prescribed by OIE lack specificity. Therefore, the investigation was initiated with the objective to enhance sensitivity and specificity of the PCR method for diagnosis. It included optimization of PCR based method which is able to detect LSD and its differentiation with Goat Pox and Sheep pox Viruses. The method was validated by isolation of DNA from 18 samples of LSDV affected animals, 2 samples from apparently healthy animals, goat pox, sheep pox and camel pox virus. The method efficiently detected the organism from all the 18 sample of LSDV affected animals and reacted negatively with 2 samples from healthy animals, sheep pox, goat pox and camel pox. The method offered remarkable limit of detection of 17.78 pg of DNA template.

Keywords: Viral attachment protein, p32 gene, PCR, *Capripoxvirus*, lumpy skin disease (LSD), lumpy skin disease virus (LSDV)

Introduction

The viral diseases have always posed challenge before researchers for their prompt diagnosis and treatment plan. Such diseases are deleterious to humans as well as animal health. The infectious diseases in dairy or poultry animals pose threat to economy and food security in the Nation [1, 2]. One such viral disease of dairy animals attracted attention of several researchers due to its current epidemic in the Asian countries [3, 32]. The LSD has recently become rampant and caused havoc in several northern states of the country [4, 34]. The causative agent of this disease comes under family Poxviridae, sub-family Chordopoxvirinae and genus Capripox viruses with other related viruses namely, Sheep Pox and Goat Pox virus [5]. The disease is transmitted through biting flies, mosquitoes and ticks. The disease occurs with pyrexia of 41 °C, swollen superficial lymph nodes and distinct lesions on skin.

The disease was first reported in Zambia in the year 1929. Spreading from Southern Africa to North, entering Middle East by 1980, sporadic outbreaks were reported. By 2012, LSDV invaded Iran, Iraq, Jordan, Turkey and Russia. By 2015 mainland Europe was affected. It was reported for the first time in India from Khairbani village of the Orissan Mayurbhanj district on August 12, 2019 [6].

The prompt treatment plan could be initiated with early diagnosis of the disease. The clinical diagnosis of LSDV relies on Enzyme Linked Immunosorbant Assay [7, 8], Immunoperoxidase Monolayer Assay [9], Virus Neutralization test [10], Indirect Fluorescent Antibody Test [11] and Agar Gel Immuno Diffusion Assay (OIE, 2017) [12]. The virus confirmation can be performed by electron microscopy [13], virus isolation [14], Immunohistochemistry [15], isothermal gene amplification [16], CRISPR based detection [17] and Polymerase Chain Reaction [18-26].

However, the serological tests fail to differentiate closely related sheep pox, goat pox and LSDV organisms. Since, PCR being most preferred method of disease diagnosis, OIE has recommended primers and protocol which can be followed in laboratory. The PCR reaction as recommended by OIE treats all the members of genus Capripoxviruses similarly, making it inconvenient to differentiate between the closely related pathogens. Therefore, the present study was envisaged to investigate an alternate PCR based diagnostic strategy with improved sensitivity and specificity. The gene p32 has been selected in the studies, which codes for antigenic structural protein. It is similar to the p35 protein of the vaccinia virus, which is expressed on the mature intracellular virion's envelope [7]. The synthesis of the 32 kd protein starts after the onset of DNA replication and is reversibly inhibited by cytosine arabinoside indicating that it is a late protein [27]. The synthesis of this protein occurs in membrane-free ribosomes without involving the endoplasmic reticulum [28]. The p32 antigen is a major antigenic determinant of Capripoxviruses and is structural protein present in all the strains. The skin nodule samples, nasal swabs and EDTA blood samples can be easily collected and used for LSDV detection [29].

Materials and Methods

Investigation was initiated with confirmation of clinically LSDV infected animals by already reported method, recommended by OIE [18]. Eighteen scab samples (S1- S18) from clinically infected animals and 2 skin scraping (S18-S19) from apparently healthy animals were collected in Dulbecco's Modified Eagle Medium (DMEM). Approval of the Institutional Animal Ethics Committee (IAEC) was taken for the collection of scabs from infected animals. Genomic DNA was extracted from the scab samples through spin column method by QIAamp DNA Mini kit, following manufacturers' protocol. Isolated DNA was subjected to PCR amplification. The 25 µl reaction mixture using 12 µL 10X Dream Taq Green Buffer, 1 µL each of forward and reverse primers (20 pmole/ µL), with 2 µL (50-100 ng/µL) DNA template was prepared for thermocycler reaction. The PCR condition included a denaturation step of 3 min at 95 °C followed by 36 cycles of amplification (45 sec at 95 °C, 50 sec at 50 °C and 1 min at 72 °C) and a final extension step at 72 °C for 10 min. Additionally, the samples were confirmed for LSDV using previously reported PCR method [26] with the conditions of denaturation step of 5 min at 95 °C followed by 35 cycles of amplification (30 sec at 95 °C, 30 sec at 52 °C and 80 sec at 72 °C) and a final extension step at 72 °C for 10 min.

To carry out the investigation further, the p32 sequences available at NCBI database were utilized to design the primers. Two pairs of LSDV p32 gene specific primers namely, SB01 and SB02 were designed and attempt was made to optimize the PCR conditions. The sequences of primers are: SB01 Fw- CCAATCGTTGGTCGCGAAAT

Rv- ATCCGCATCGGCATACGATT
SB02 Fw- GGAAATCGTATGCCGATGCG
Rv- TCATATCCCCCTGTGTACGA

The primer pair SB01 were designed for initial screening of Capripox virus infected animals with an expected product size of 232 bp. The PCR condition followed for SB01 primers, included a denaturation step of 7 min at 96 °C followed by 38 cycles of amplification (1 min at 96 °C, 1 min at 52 °C and 1 min at 72 °C) and a final extension step at 72 °C for 10 min. The DNA isolated from confirmed camel pox (CPV) infected animal, sheep pox vaccine (SPV) and goat pox vaccine (GPV) were taken as negative control in PCR reaction.

For the purpose of differentiation of LSDV with GPV and SPV, LSDV p32 gene specific primers SB02 were designed with an expected product size of 303 bp. The PCR condition followed for SB02 primers were same as described for SB01 except the annealing temperature of 66 °C was utilized. The PCR products were analyzed on 1.8 % agarose gel electrophoresis with 100 bp molecular marker. The PCR product was sent for Sanger sequencing to Eurofins Genomics India Pvt. Ltd. The p32 sequence thus obtained was subjected to phylogenetic analysis using ClustalV algorithm in MegAlign software.

Result and Discussion

PCR is the most accepted technique for diagnosis of pathogenic organism. On investigation of clinically suspected samples, one out of three clinically positive samples showed no amplification using OIE recommended PCR based method (Figure 1). These DNA samples were subjected to polymerase chain reaction for confirmation using methods reported earlier (Figure 2). All the samples of LSD as well as SPV, GPV were amplified by the earlier methods [18, 26]. The DNA samples were considered as positive in case any one of the previously reported method tested positive (Table 1).

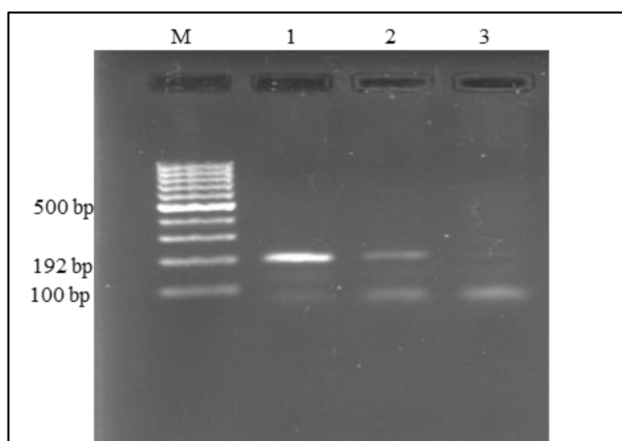

Fig 1: Gel Electrophoresis (1.8 %) of PCR amplified products of LSDV genomic DNA as template using primers as recommended by OIE for conformation. Lane M-100 bp marker, Lane 1-3: PCR amplified product (192 bp) from samples S14-S16.

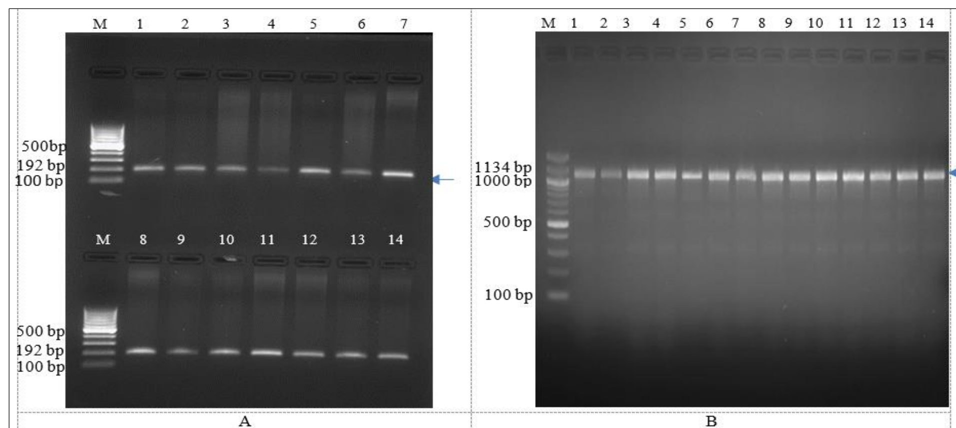

Fig 2: Amplification of LSDV genome. Lane M indicate DNA size marker of 100 bp size. Lanes 1–12, Lane 13 and Lane 14 indicate the PCR products of 12 LSD samples, SPV and GPV respectively. A and B indicates PCR amplification using OIE recommended primers and ORF011 primers (Kumar *et al*., 2021) [26], respectively.

Table 1: Result of validation studies performed to evaluate the functionality of SB02 primers

Samples (1)	OIE (2)	ORF011 (3)	Result (4)	P32 (5)
LSDV 1	+	+	+	+
LSDV 2	+	+	+	+
LSDV 3	+	+	+	+
LSDV 4	+	+	+	+
LSDV 5	+	+	+	+
LSDV 6	+	+	+	+
LSDV 7	+	+	+	+
LSDV 8	+	+	+	+
LSDV 9	+	+	+	+
LSDV 10	+	+	+	+
LSDV 11	+	+	+	+
LSDV 12	+	+	+	+
LSDV 13	+	+	+	+
LSDV 14	+	+	+	+
LSDV 15	+	+	+	+
LSDV 16	-	+	+	+
LSDV 17	+	+	+	+
LSDV 18	-	-	-	-
LSDV 19	-	-	-	-
LSDV 20	+	+	+	+
CPV	-	-	-	-
SPV	+	+	-	-
GPV	+	+	-	-

Hence, it was felt that an alternate strategy is highly required to conduct molecular diagnosis of LSDV affected animals. Therefore, the investigation was carried out with the objective of developing a PCR based diagnostic strategy for LSDV detection with improved sensitivity and to optimize the PCR conditions for specific differentiation of LSDV with GPV and SPV.

For this purpose, the p32 gene length of 969 nucleotides has been utilized for designing of primer pairs. The primer set SB01 amplified 232 bp of Capripox p32 gene (Figure 3) for initial screening. The method amplified all of the samples falling under capripox genus and no amplification was observed in negative control. To validate the functionality of the developed method for specific diagnosis of LSDV through primer set SB02, 23 samples were included in the studies.

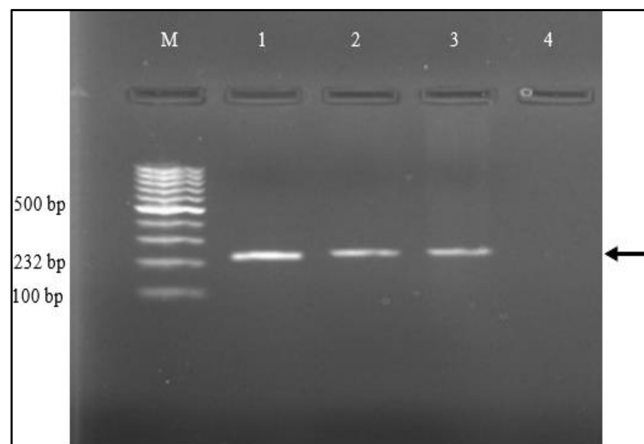

Fig 3: Amplification of Capripoxvirus genome. Lanes M indicate DNA size marker of 100 bp size. Lanes 1,2,3,4 indicate the products of size 232 bp using SB01 primers of LSDV, SPV, GPV and NTC respectively. The arrow indicates length of the desired product (232 bp)

At the annealing temperature of 66 °C, the primer set detected 18 scab samples as positive for LSDV and 2 skin healthy scraping samples along with GPV, SPV and CPV reacted negatively (Figure 4).

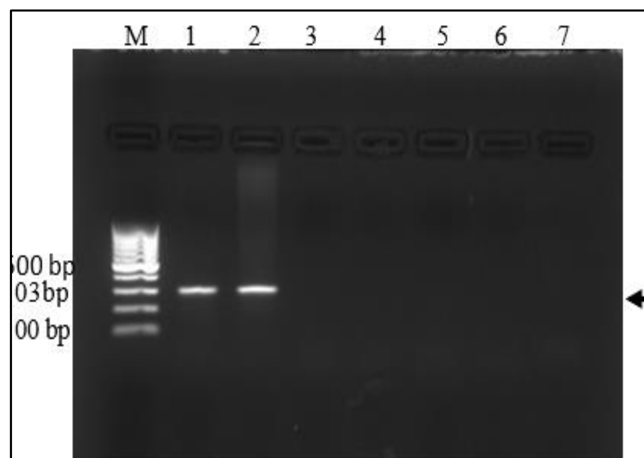

Fig 4: PCR for differentiation of LSDV, SPV, GPV and CPV at 66 °C annealing temperature. Lanes M indicate DNA size marker of 100 bp size. Lanes 1–2, Lane 3, Lane 4, Lane 5 and lanes 6–7 indicate the two LSDV samples, SPV, GPV, CPV and 2 skin scrapping's of healthy animals, respectively. The arrow indicates length of the desired product (303bp)

The sensitivity and specificity [30] was calculated using the formulae as follows-

Sensitivity = true positives/ true positives + false negatives x 100 = 18 / 18+0 x 100 = 100 %

Specificity = true negatives/ true negatives + false positives x 100 = 5 / 5+ 0 x 100 = 100 %

On comparison with previous reported methods (Gari et al., 2008; El-Khabaz et al., 2014; Samojlovic et al., 2019; Babiuk et al., 2019; Jiang et al., 2022), the validation studies of the developed method present higher sensitivity of 100 %, specificity of 100 % with positive predictive value of 100 % and negative predictive value of 100 % for LSDV diagnosis [10,11,17,31,33]. Serially diluted LSDV DNA template in PCR experiment showed limit of detection (LOD) of 17.78 pg (Figure 5).

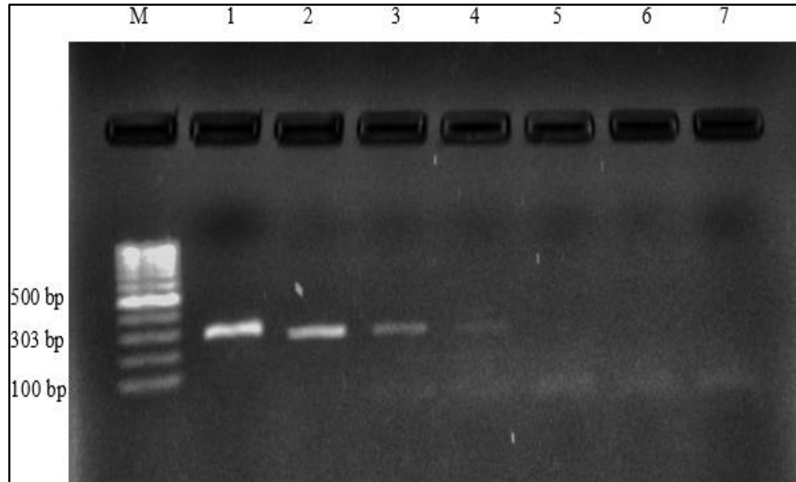

Fig 5: PCR for investigation of Limit of detection (LOD). Lanes M indicate DNA size marker of 100 bp size. Lanes 1–7 indicate serial 10 fold dilution of viral DNA sample (template), starting with a concentration of 17.78 ng. The arrow indicates length of the desired product (303 bp)

The sequence of amplified product was submitted in NCBI database with an Accession Number- OR338843. The sequence was further aligned with reported p32 sequences from NCBI, the phylogenetic tree represents close similarity of SPV gene sequence reported from China in year 2013 (Figure 6).

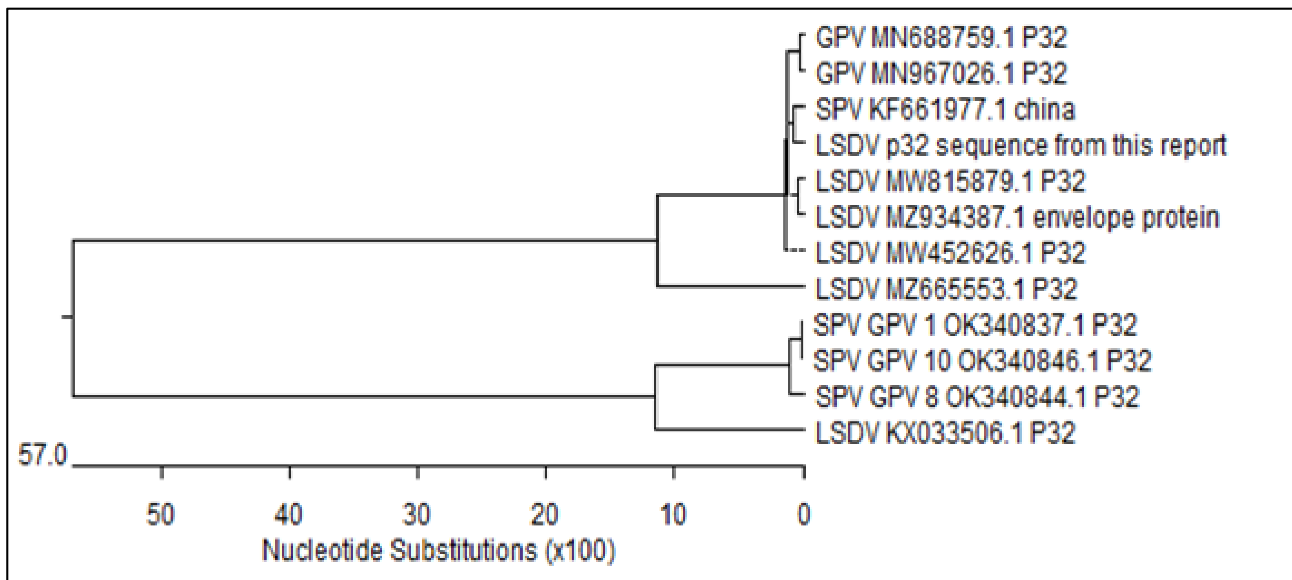

Fig 6: Phylogenetic analysis

Conclusion

The present investigation was planned with the objective of improvement in sensitivity and specificity of the previously reported PCR methods. An attempt was made to develop newer PCR based method. For this purpose, one of the primers namely SB01, can be used for initial screening of Capripox viruses and SB02 can be used for specific diagnosis of LSDV and its differentiation from SPV, GPV and CPV with decent performance.

Acknowledgments

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