International Journal of Advanced Biochemistry Research

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; SP-8(1): 960-964
www.biochemjournal.com
Received: 12-10-2023
Accepted: 16-11-2023

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Diagnosis of lumpy skin disease based on RNA polymerase subunit 30 gene polymerase chain reaction

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DOI: https://doi.org/10.33545/26174693.2024.v8.i1Sm.486

Abstract
The prompt diagnosis and treatment of viral disease in dairy and poultry animals has always been a great challenge before a clinician. Any delay in such operations may lead to economic losses and destruction to dairy, poultry related food industries and pose a threat to food security in the country. In recent times, the Lumpy Skin Disease (LSD) has become rampant and caused havoc in several Asian countries and northern states of India with loss of more than Rs. 600 Cr. The disease is caused by Lumpy Skin Disease Virus (LSDV) from Poxviridae family and Capripox virus genus, with other related viruses namely, Sheep Pox and Goat Pox viruses. It is transmitted through several arthropods *viz.* biting flies, mosquitoes and ticks. It is marked with high fever of 41 °C, inflammation in superficial lymph nodes and distinct scab lesions on skin. The clinical diagnosis of LSDV relies on ELISA, Indirect Fluorescent Antibody Test (IFAT), AGID, virus confirmation by transmission electron microscopy, virus isolation and PCR. OIE has recommended PCR based method for screening of LSD diagnosis. The present study was envisaged to investigate an alternate PCR based diagnostic strategy with improved sensitivity and specificity. The RNA Polymerase subunit gene (RPO30) has been selected for the investigation. In this regard, the target nucleotide sequence of 602 bp length has been utilized for designing of two primers namely, SR01 and SR02. The SR01 was found to be capable of screening of Capripox viruses. The nucleotide alignment of RNA polymerase gene from other Capripox viruses reveals significant differences at several positions. Therefore, such non-conserved portion was utilized for primer designing with the objective to differentiate among closely related members of Capripox viruses. The PCR reaction was optimized for amplification of the gene with annealing temperature of 66 °C. No PCR amplification was observed with GPV, SPV and CPV as source of target DNA. The validation of the method was conducted on DNA isolated from sixteen scab samples. The developed method offers highest sensitivity, specificity with excellent positive predictive and negative predictive value for LSDV diagnosis. The PCR experiment with serially diluted LSDV DNA template showed decent limit of detection (LOD) of 50.7 pg.

Keywords: Lumpy skin disease, RNA polymerase, polymerase chain

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 country [1, 2]. One such viral disease of dairy animals attracted attention of several researchers due to its current epidemic in the Asian countries [3]. The Lumpy Skin Disease (LSD) has recently become rampant and caused havoc in several northern states of the country [4]. The causative agent of this disease, Lumpy Skin Disease Virus (LSDV) comes under family Poxviridae, sub-family Chordopoxvirinae and genus Capripox viruses with other related viruses namely, Sheep Pox and Goat Pox viruses [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 of the Orissan Mayurbhanj district in 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 [12]. The virus confirmation can be performed by electron microscopy [13], virus isolation [14] and Immunohistochemistry [15], isothermal gene amplification [16], CRISPR based detection [17], Polymerase Chain Reaction [18, 19, 20, 21, 22, 23, 24]. PCR being most preferred method of disease diagnosis, OIE has recommended primers and protocol which can be followed in laboratory. Therefore, the present study was envisaged to investigate an alternate PCR based diagnostic strategy with improved sensitivity and specificity using scab samples. The 606 nucleotides long viral RPO30 gene has been selected in the studies, which codes for RNA polymerase subunit 30 exhibiting nucleotidyl transferase activity.

Materials and Methods

The RPO30 sequence (Accession number MT228884.1) from NCBI database was utilized to design the primers. Two LSDV RPO30 gene specific primers namely,
SR01 (Fw: ATAACCTCCCATGCCCTGAGT; Rv: GGTGGTTCATCTGCTGCTCTT) and SR02 (Fw: ACGTACGACAAATGGAACAGA; Rv: TTTTACACTCAGGGCATGGGA) were designed and attempt was made to optimize the PCR conditions. For initial screening of Capripox virus infected animals, Capripox genus RPO30 gene specific primers (SR01) was designed with an expected product size of 79 bp. For differentiation of LSDV with GPV and SPV, LSDV RPO30 gene specific primers (SR02) were designed with an expected product size of 170 bp.
Eighteen samples were used for validation of functionality of the PCR primers. Out of these, sixteen scab samples (S1-S16) from clinically infected animals were collected in DMEM and sheep pox virus (SPV) and goat pox virus (GPV) were taken as negative control in PCR reaction. DNA isolation was carried out from all the eighteen samples. Genomic DNA was extracted from the scab through spin column method by QIAamp DNA Mini kit, following manufacturers' protocol. The clinical samples were confirmed for presence of LSDV. In this regard, PCR was performed for the amplification of viral ORF011 gene specific primers [23]. Isolated DNA was subjected to amplification using conditions as described: the 25 µl reaction mix from 1 X *Taq* polymerase (#K1081, Thermo Scientific), forward and reverse primers (0.8 pmoles each) with around 50 ng DNA template was prepared for thermocycler reaction. The PCR condition followed included a denaturation step of 7 min at 96 ˚C followed by 38 cycles of amplification (1 min at 96 ˚C, 1 min at 66 ˚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. The PCR products were analyzed on 1.8% agarose gel electrophoresis with 100 bp molecular marker.

Result and discussion

The RPO30 gene length of 606 bp has been utilized for designing of primer pair with the objective to improve sensitivity and selectivity of the LSDV diagnosis and to differentiate LSDV with GPV and SPV. The primer set SR01 amplified 79 bp of Capripox p32 gene (Figure 1) for initial screening. The primers decently amplified RPO30 from all three members of Capripox virus at varying temperature conditions between 50-58 °C (Figure 1a-c). To validate the functionality of the developed method for specific diagnosis of LSDV through primer set SR02, 19 samples were included in the studies. These DNA samples were subjected to polymerase chain reaction for confirmation using methods reported earlier. At the annealing temperature of 66 °C, the primer set detected scab samples as positive for LSDV along with Goat Pox Virus (GPV) and Camel Pox Virus (CPV) reacted negatively (Figure 2). However, a faint band is exhibited by Sheep Pox virus (SPV) can be treated as negative on the basis of vague brightness as compared to the LSDV counterparts. On tabular compilation of the results obtained with 16 LSDV DNA samples and 2 CPV, SPV and GPV samples, tested with previous methods of OIE (2017) and Kumar *et al.* (2021), the DNA samples were considered as positive in case any one of the methods tested positive [18, 23, 25] (Table 1). The previous methods tested positively for SPV and GPV. However, the present method differentiated SPV and GPV with LSDV samples. Therefore, the sensitivity and specificity [26] 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%

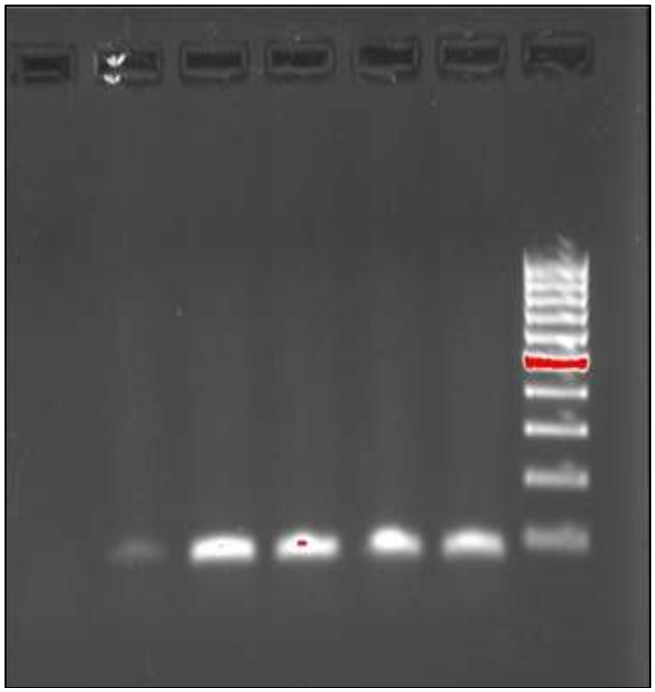

Fig 1a: Gel Electrophoresis (1.8%) of PCR amplified products (79 bp) of LSDV genomic DNA S1 as template using designed primer SR01.

Lane 1: NTC
Lane 2: at Annealing temperature 50 °C
Lane 3: at Annealing temperature 52 °C
Lane 4: at Annealing temperature 54 °C
Lane 5: at Annealing temperature 56 °C
Lane 6: at Annealing temperature 58 °C
Lane M: 100 bp marker

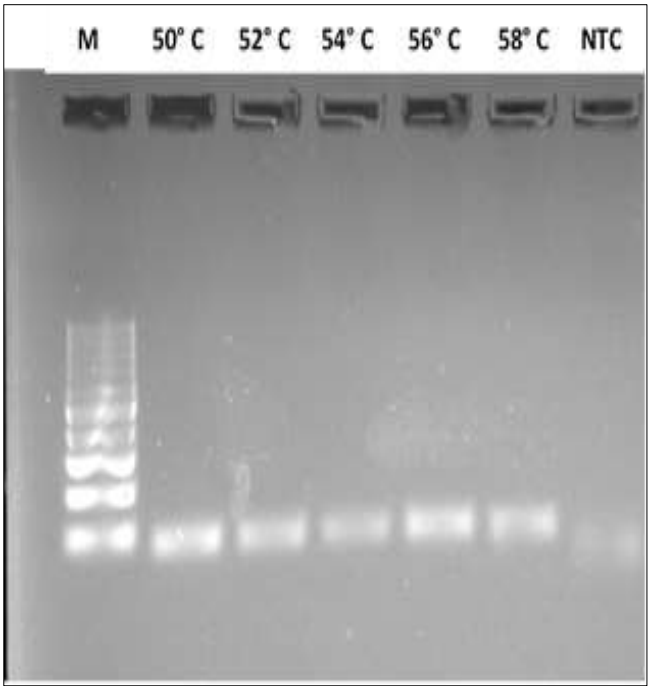

Fig 1b: Gel Electrophoresis (1.8%) of PCR amplified products (79 bp) of SPV genomic DNA as template using designed primer SR01.

Lane M: 100 bp marker.
Lane 1: at Annealing temperature 50 °C
Lane 2: at Annealing temperature 52 °C
Lane 3: at Annealing temperature 54 °C
Lane 4: at Annealing temperature 56 °C
Lane 5: at Annealing temperature 58 °C
Lane 6: NTC

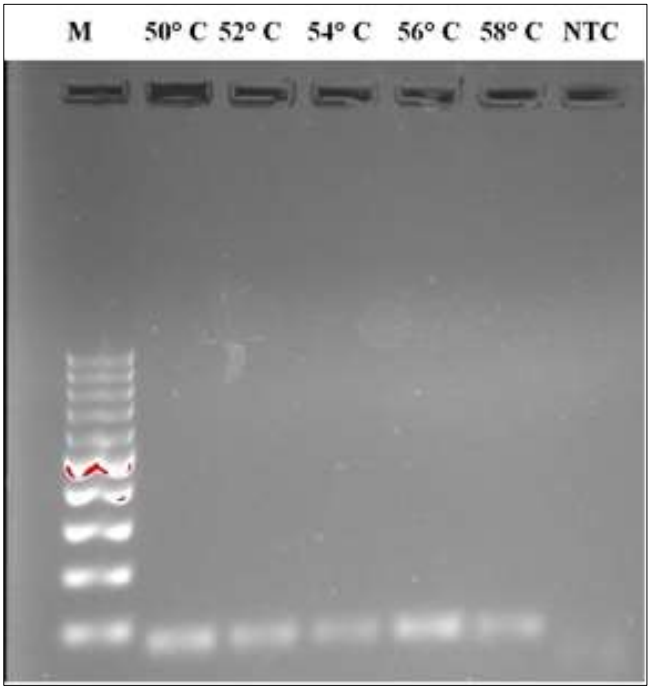

Fig 1c: Gel Electrophoresis (1.8%) of PCR amplified products (79 bp) of GPV genomic DNA as template using designed primer SR01.

Lane M : 100 bp maeker.
Lane 1: at Annealing temperature 50 °C
Lane 2: at Annealing temperature 52 °C
Lane 3: at Annealing temperature 54 °C
Lane 4: at Annealing temperature 56 °C
Lane 5: at Annealing temperature 58 °C
Lane 6: NTC

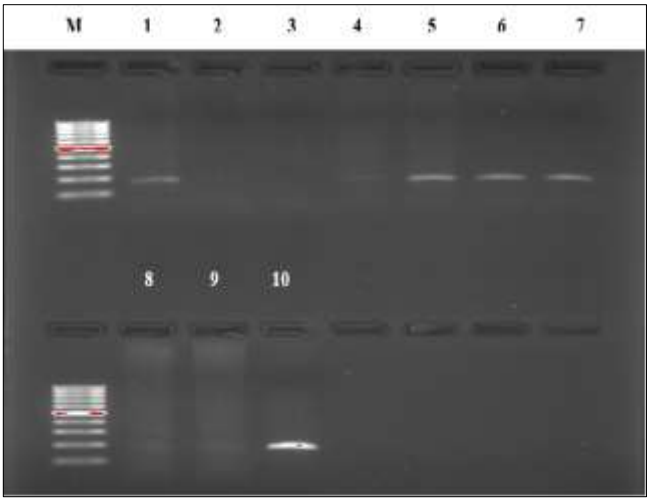

Fig 2: Gel Electrophoresis (1.8%) of PCR amplified products (170 bp) of genomic DNA as template using designed SR02 primer at annealing temperature 66 °C

Lane M : 100 bp marker.
Lane 1: LSDV.
Lane 2: SPPV.
Lane 3: GTPV
Lane 4-7: LSDV
Lane 8: CPV.
Lane 9: CPV.
Lane 10: LSDV

Table 1: Validation studies performed to evaluate the functionality of SR02 primers

Samples	OIE, 2017	Kumar *et al*., 2021 [24]	Bishnoi *et al*., 2024 [20]	This report
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	+	+	+	+
CPV 1	-	-	-	-
CPV 2	-	-	-	-
SPV	+	+	-	-
GPV	+	+	-	-

In the current study the high sensitivity of PCR (100%) in detecting the LSDV DNA in skin nodular samples correlate very well with (El-Khabaz *et al*. 2014) [27] and this may be attributed to the viral tropism to skin tissues and its persistence in high concentration as PCR with blood samples yielded no result. The accuracy of IFAT in LSD detection showed 92% sensitivity and 88% specificity (Gari *et al*., 2007) [28]. The specificity of VNT and ELISA was 100% and 99.2%, respectively (Samojlovic *et al*., 2019) [29]. On site RPA-Cas12a-fluorescence assay showed a diagnostic sensitivity of 96.3% and specificity of 92.31% [17]. Thus on comparison with previously reported methods, the method showed remarkable sensitivity and

specificity as compared to the benchmark tests of virus neutralization method [29] and genotyping methods (Gelaye *et al*., 2013) [30]. Serially diluted LSDV DNA template in PCR experiment showed limit of detection (LOD) of 17.78 pg (Figure 3).

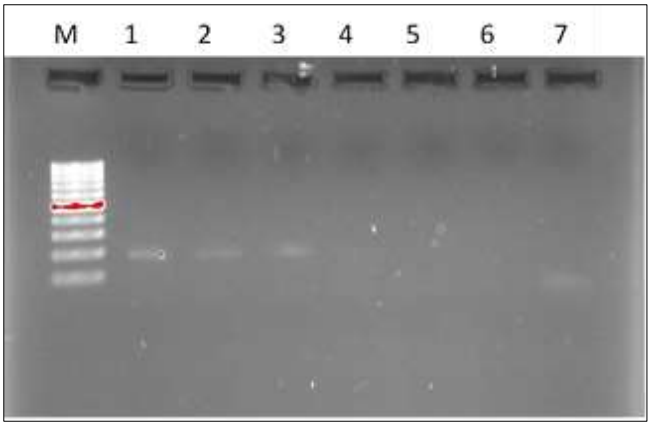

Fig 3: Limit of detection- Gel Electrophoresis (1.8%) of PCR amplified products of LSDV genomic DNA as template using SR02 primers and varying template concentration.

Lane M: 100 bp marker.
Lane 1: 6.13 ng
Lane 2: 557 pg
Lane 3: 50.7 pg
Lane 4: 4.61 pg
Lane 5: 419 fg
Lane 6: 38 fg
Lane 7: NTC

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 with designing of newer PCR primers. Out of 2 PCR primers designed SB01 can be used for initial screening of Capri pox viruses, SB02 can be used for specific diagnosis of LSDV and its differentiation from SPV, GPV and CPV with remarkable performance.

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