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LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY FOR RAPID DETECTION OF ORF VIRUS INFECTION IN SHEEP AND GOATS IN KARNATAKA.

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ABSTRACT The Loop mediated isothermal amplification (LAMP) assay was standardized using six primers to amplify B2L gene of Orf virus (ORFV). Conventional polymerase chain reaction (PCR) was also standardized targeting B2L gene for detection of ORFV. A total of 70 scab samples were collected from sheep and goats showing symptoms of Orf disease. The PCR screening of 70 scab samples revealed 60 percent positivity for ORFV. The LAMP assay was standardized using Hydroxynapthol blue dye at the final working concentration of 200M in the reaction mixture for the visual detection of ORFV in PCR positive scab samples. The successful amplification was indicated by a colour change from deep blue to light blue. All the PCR positive samples were also found positive with LAMP assay. Although the LAMP reaction had equal sensitivity to the PCR test, it is considered superior because of its simplicity to carryout in field conditions and at quarantine inspection places.

KEYWORDS : Orf virus, LAMP, PCR, Sheep & Goats

Introduction

Orf, also known as contagious ecthyma, sore and scabby mouth disease, contagious pustular dermatitis, infectious pustular dermatitis, infectious labial dermatitis and ovine pustular dermatitis, is an endemic, acute debilitating, eruptive skin, non-systemic and often self-limiting disease of domestic animals, most commonly in sheep and goats of worldwide and also affects human beings. Young animals are severely affected, lesions are commonly seen around mouth, lips, ears, eyes and nose, due to the property of the virus that infects the epithelial cells of the skin (Bora et al., 2011).

The Orf disease is the result of infection with Orf virus (ORFV), a pox virus of the genus parapoxvirus (PPV) represents one of the nine genera in the Chordopoxvirinae subfamily in the family Poxviridae, which include the ORFV, the Bovine papular stomatitis virus (BPSV), Pseudo cowpox virus (PCPV), Parapoxvirus of red deer in New Zealand and PPV of the gray seal. Other members of the genus include a seal pox virus (Nettleton et al., 1995) and viruses that infect red squirrels (Sainsbury and Gurnell,1995) and camels (Dashtseren et al., 1984).

Orf virus is robust in dry environment, where it can survive for months or even years, but in cold and wet conditions its life span may be shorter (McKeever and Reid, 1986). The ORFV infects via broken, scarified or otherwise damaged skin and replicates in epidermal cells. Clinically, Orf disease progresses from erythema to macule / papule / vesicle to pustule then scab. Primary lesions can be severe and proliferative, but generally resolve within 6weeks. Re infections are less severe and resolve more rapidly than primary ones, usually within 2 weeks. Virus may shed in scabs, and there is no evidence of systemic spread. Although it is clear that Orf outbreaks occur through contact with infectious materials in the environment, the possibility that the virus may be maintained with in a herd or flock by sub-clinical infections of periodically damaged skin has not been rigorously explored (McKeever et al., 1987).

Diagnosis of Orf infections in general based on clinical signs, virus isolation and electron microscopy which are commonly used along with serological tests. However, these methods are laborious, time consuming and in some cases not effective. For example, virus isolation can be unsuccessful at all times. Polymerase chain reaction (PCR) based detection of the B2L gene of ORFV which encodes a major envelope protein, has been the most widely used approach for diagnosis of orf and this method has shown good sensitivity and specificity (Inoshima et al., 2000). However, this assay requires skilled technicians and specialized instruments.

Loop mediated isothermal amplification (LAMP) is a novel method that facilitates rapid nucleic acid amplification using only simple equipment and first described as an autocycling strand displacement DNA synthesis using the Bst DNA polymerase. Bacillus stearothermophilus (Bst) DNA polymerase has two distinct activities; linear target isothermal multimerisation and amplification, and cascade rolling-circle amplification. The LAMP assay rapidly amplifies specific DNA sequence with high sensitivity under isothermal conditions and was first developed by Notomi et al. (2000). The LAMP assay is a powerful innovative gene amplification technique emerging as a simple, rapid diagnostic tool for early detection and identification of microbial diseases. This technique simplifies the instrumentation and reduces the electricity requirements by eliminating the need for thermal cycler (Njiru, 2012). Loop mediated isothermal amplification products can easily be detected by the naked eye due to the formation of magnesium pyrophosphate, a turbid - white by-product of DNA amplification that accumulates as the reaction progress. The objective of the present study was to standardize the LAMP assay and compared with conventional PCR for the rapid detection of orf virus in clinical samples.

Materials and methods

Collection of samples

The scab samples were collected from high density sheep and goats rearing districts in Karnataka state, India. The scabs around the mouth lesions (Fig.1) were collected aseptically. A total of 70 scab samples were collected from sheep (46 samples) and goats (24 samples) showing symptoms of Orf disease in eight districts of Karnataka with dense sheep population.

Fig.1: Gross lesions around mouth (proliferative form of Orf) in sheep and goat

DNA extraction

The DNA was extracted from the clinical samples using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol.

Conventional PCR

The published primers for B2L gene of ORFV by Hosamani et al., 2006 were synthesized at M/s Chromous biotechnologies, Bengaluru (Table 1).

Table 1: List of published primers used for Polymerase chain reaction (Hosamani *et al*., 2006)

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Name of the primers	Nucleotide sequences (5'-3')	Primer location	Amplicon size
OVB2LF1	TCCCTGAAGCCCTATTA TTTTTGTG(25mer)	560-583	1137bp
OVB2LR1	GCTTGCGGGCGTTCGG ACCTTC (22mer)	1138-1115	

The Polymerase chain reaction tubes containing the mixture were tapped gently, spun briefly and the components were transferred to thermal cycler (Eppendorf master cycler, Germany). The annealing temperature was optimized for 52°C. The final PCR protocol used for the two pairs of primers was standardized for the conditions as initial denaturation step of 94°C for 3 min followed by 29 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min and final extension of 72 °C for 7 min. An aliquot (5 ml) of PCR product was analyzed by 1.0 % agarose gel electrophoresis to visualize the PCR amplicons. The gene amplicons were gel purified using MinElute gel extraction kit (QIAGEN) as per manufacturer's protocol. The non-template control consisted of sterile water instead of DNA template, while positive control consisted of DNA from reference virus.

LAMP primers

The published primers for B2L gene of ORFV by Su-ming et al. (2009) were synthesized at M/s Chromous biotechnologies, Bengaluru (Table 2).

Table 2:Details of LAMP Primers for the detection of Orf virus

Name of Primers	Oligonucleotide sequences (5'-3')	Product length (bp)
Forward outer (F3)	GCGCAGGGCAACATGA	16
Reverse outer (B3)	GCTCGTCAGCGTCCTT	16
Forward inner, FIP (F1c + F2)	GTTGCAGCAGAAGCTGCAGATG GCA CCCTCGACTGCTTCAC	41
Reverse inner BIP (B1c + B2)	GGACAAGCTATGCACGCTCGCC TTGCTCTGCACGTCCA	38
Forward loop (LF)	TTCTTCGCGGACTCGGC	17
Reverse loop (LB)	TAAGGAGGGCGTCGACGTCAC	21

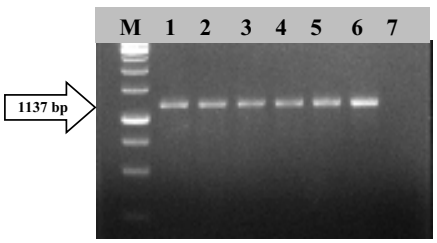
Optimization of Loop mediated isothermal amplification assay conditions

Loop mediated isothermal amplification reactions were optimized as described by (Su-ming et al., 2009). An evaluation of the effects of different concentration of reaction mixture were carried out to optimize the LAMP reaction. The LAMP reaction was optimized at a volume of 25 µl containing 1X Thermopol buffer, 12 mM MgSO4, 0.6 M Betaine, 1.2 mM dNTPs, 8U Bst polymerase, 0.2 µM of each of the F3 and B3 primers, 1.6 µM of each of the FIP and BIP primers, 0.8 µM of each of the LoopF and LoopB primers, 200M HNB disodium and 1 µl of extracted DNA as template. The amplification was performed at 67°C in a laboratory water bath for 75min. A control containing no template was included in each test as the negative control. The LAMP products were visualized with the naked eye. They were also separated on agarose gel electrophoresis using 2.2 per cent agarose in 0.5X TBE buffer, followed by staining with ethidium bromide and was visualized under UV light and documented by gel documentation system.

Results & Discussion

After standardization of different components of PCR, DNA extracted from 70 scab samples were subjected to PCR for detection of B2L gene of ORFV. In the present study, out of 70 samples from 27 outbreaks, 42 samples (60%) were positive for the amplification of B2L gene of ORFV (Fig. 2). In Tumkur district, out of 21 samples, 12 were positive (57.1%) by PCR, in Chitradurga district, out of 33 samples, 17 were positive (52 %) by PCR. In other districts of Davangere, Bagalkot, Bellary, Hassan, Bengaluru and Gadag, the per cent positivity of the samples were 100 per cent, 100 per cent, 100 per cent, 66.6 per cent, 66.6 per cent and 0 per cent respectively.

Fig. 2: PCR amplification of B2L gene of Orf virus



Lane M: 250bp DNA ladder
Lane 1: ORFV KVAFSU VMC -05
Lane 2: ORFV KVAFSU VMC -20
Lane 3: ORFV KVAFSU VMC -51
Lane 4: ORFV KVAFSU VMC-59

Standardization of Loop mediated isothermal amplification for Orf virus detection

The LAMP was standardized using six primers published by Su-ming et al. (2009) to amplify B2L gene of ORFV. The LAMP reaction conditions were optimized to a total volume of 25µl reaction mixture at 67°C for 75 min using Bst DNA polymerase.

Optimization of Loop mediated isothermal amplification Assay condition

All the possible variables were tested. The LAMP assay was optimized as described by Su-ming et al. (2009) with some modifications in concentration of MgSO4, Betaine, dNTPs, nd Temperature and Incubation time (Table 3). The PCR positive DNA samples were used to optimize the LAMP assay. The negative controls (cDNA of CSFV and bacterial agents like Staphylococcus aureus) were included for each LAMP reaction. The LAMP reaction was performed at 670C in a laboratory water bath for 75 min.

Table 3: Optimization of Loop mediated isothermal amplification assay condition

Reaction component/Parameter (units)	Range
MgSO4 (mM)	2, 4, 6, 8, 10 and 12
Betaine (M)	0.2, 0.4, 0.6 and 0.8
dNTP (mM	0.2, 0.4, 0.6, 0.8, 1.0 and 1.2
Assay temperature (oC)	63, 65 and 67
Incubation time (min.)	60, 65, 70 and 75

Visual detection of the Orf virus Loop mediated isothermal amplification products by application of Hydroxynapthol blue

The hydroxy-napthol blue, a metal chelating agent was incorporated in the ORFV LAMP assay for better visualization of the amplified product. The final working concentration of 200M HNB in the reaction mixture was optimized to achieve the desirable results. The HNB

induced color changed from deep blue in negative reactions to light blue in positive samples when observed under visible light with naked eye (Fig. 3).The LAMP products were also visualized-ladder like banding pattern after subjecting to 2.2 per cent agarose gel electrophoresis (Fig.4). All the PCR positive samples were also subjected for optimized LAMP assay and were found positive.

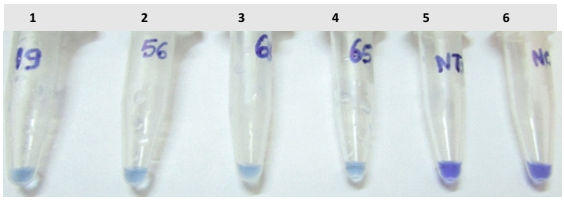
Standardization of Loop mediated isothermal amplification (LAMP) for detection of Orf virus

Loop mediated isothermal amplification is a novel method that facilitates rapid nucleic acid amplification using only simple equipment i.e water bath and first described as an autocycling strand displacement DNA synthesis using the *Bst* DNA polymerase (Notomi et al., 2000). *Bst* DNA polymerase has two distinct activities first, linear target isothermal multimerisation and amplification, secondly, cascade rolling-circle amplification (Hafner et al., 2001). A different LAMP amplification pattern appeared as a result of linear target isothermal multimerisation and amplification, as LAMP primers and target DNA seem to randomly multimerize (Kuboki et al., 2003).

The most extensively used diagnostic methods for detection of ORFV are cell culture and PCR. These techniques, however, require a relatively long time to obtain results and are not practically feasible for field application, because of the equipment and expertise needed to conduct the assays. Moreover, the Taq DNA polymerase used in the PCR assay is easily inactivated by tissue and blood-derived inhibitors such as myoglobin, heme-blood protein complex and immunoglobulin-G (Akane et al., 1994; Johnson et al., 1995; Belec et al., 1998 and Al-Soud et al., 2000). The LAMP assay mostly depends on the specificity of the primer sets. Therefore, primers were selected from the highly conserved region of B2L gene of the ORFV, this DNA region has been routinely chosen as the detection targets based on sequence alignments of published strains. This region is chosen in order to ensure adequate fidelity of the assay and reduce interference due to genetic mutation. Betaine was used in the LAMP reaction mixture to reduce base stacking (Rees et al., 1993). Magnesium sulphate concentration and the ratio of outer and inner primers have the most profound effect on LAMP assay because Magnesium sulphate is a key cofactor for DNA polymerase activity and the ratio of outer and inner primers affects the initial step in the formation of stem-loop DNA and to increase not only the overall rate of reaction but also target selectivity by significantly reducing amplification of irrelevant sequences (Notomi et al., 2000). The LAMP assay has numerous advantages over the systems presently in use. It does not require a thermal cycler because reactions could be carried out in a heat block or ordinary water bath under isothermal conditions. Unlike PCR, no repetitive denaturing and annealing steps are needed and the inhibitory effects from substances in samples are largely eliminated (Nagamine et al., 2001 and Kaneko et al., 2007).

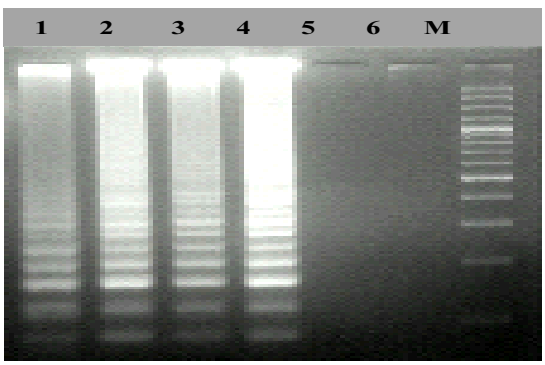
In the present study, LAMP was standardized for the detection of ORFV from the clinical samples which were taken from the naso-oral lesions of affected sheep and goats. Pilot experiments were conducted to analyze the various factors affecting the LAMP assay conditions like buffers, magnesium sulphate, betaine, dNTPs, temperature and time, by keeping the constant ratio (1:8) of LAMP primers. A set of six published primers including two inner primers (FIP/BIP), two outer primers (F3/B3) and two loop primers (LF/LB) were used for LAMP assay. The LAMP reaction conditions were optimized to 25µl for detection of ORFV in 75 min at 65°C using Bst DNA polymerase and HNB dye was added to the optimized master-mix at a final concentration of 200M, prior to reaction. Successful amplification was indicated by a color change from deep blue to light blue (Fig.3). This will facilitate the rapid screening of samples without the use of agarose gel electrophoresis. Hydroxynapthol blue is the most acceptable solution for field-based screening of LAMP amplification products as it is both cost effective and does not require opening the tubes after the reaction is completed, unlike the picogreen dye (Song et al., 2013). However, gel electrophoresis with 2.2 per cent agarose and staining with ethidium bromide was also used to visualize the LAMP products where ladder like banding pattern was obtained (Fig.4). These finding were in accordance with the Mori et al., (2001) Iwamoto et al., (2003) and Su-Ming et al., (2009). Further all the PCR positive samples were also found positive with LAMP. Although the LAMP reaction had equal sensitivity to the PCR test, it is considered superior because of its simplicity to carryout in most situations where a rapid diagnostic method is required under field conditions and at quarantine inspection places. The whole procedure is very simple and rapid wherein the amplification can be completed within one hour under isothermal conditions by incubating all the reagents in a single tube in an ordinary water bath. These findings are in agreement with the Goto et al., (2009) and Guangxiang et al., (2013).

Fig. 3:Visualization of Loop mediated isothermal amplification results by naked eye with Hydroxynapthol blue



Tube 1: ORFV KVAFSU VMC -19
Tube 2: ORFV KVAFSU VMC -56
Tube 3: ORFV KVAFSU VMC -60
Tube 4: ORFV KVAFSU VMC -65
Tube 5: Non Template Control
Tube 6: Negative Control (cDNA of Classical swine fever virus (CSFV)

Fig. 4: Agarose gel electrophoresis of Loop mediated isothermal amplification products



Lane 1: ORFV KVAFSU VMC -19,
Lane 2: ORFV KVAFSU VMC -56
Lane 3: ORFV KVAFSU VMC -60,
Lane 4: ORFV KVAFSU VMC -65
Lane 5: Negative Control (cDNA of Classical swine fever virus)
Lane 6: Non Template Control
Lane M: 100bp DNA ladder

The risk of cross-contamination in LAMP assay is minimal because only a single step is used. In addition, the probability of an incorrect target being mistakenly amplified is rare since a successful reaction needs six independent primers concomitantly to match their respective regions in targets. As a result, only ORFV DNA is amplified among the other viral and cellular nucleic acids present in the sample (En et al., 2008). The LAMP assay was also used for the detection of rabies virus by Nithinprabhu (2014) who also used HNB dye for direct visualization of LAMP products. The LAMP method has a greater potential for monitoring the prevalence of Orf, and it could prove to be a powerful tool for current diagnostic method as also opined by Song et al. (2013). The LAMP assay has been used to amplify both DNA and RNA viruses (Foot and mouth disease virus by Dukes et al., 2006; Phytoplasma in plants by Tomlinson & Boonham, 2008; Pseudorabies by En et al., 2008). This approach was found to be an excellent tool with high sensitivity, high specificity and fast turnaround time.

Conclusion

Loop mediated isothermal amplification (LAMP) is a novel method that facilitates rapid nucleic acid amplification and having greater potential for monitoring the prevalence of Orf virus infection in sheep and goats and it could prove to be a powerful tool for current diagnostic method.

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