Standardization of loop-mediated isothermal amplification for detection of *D. nodosus* and *F. necrophorum* causing footrot in sheep and goats

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**Abstract**

The loop-mediated isothermal amplification (LAMP) was standardized for rapid detection of *Dichelobacter nodosus* and *Fusobacterium necrophorum*. A total of 250 foot swabs were screened from sheep (200) and goats (50) from different districts of Rayalaseema, viz., Chittoor, Nellore, Kadapa, and Anantapur. Out of 250 samples, 75 (30.0%) and 85 (34.0%) were positive for *D. nodosus* and *F. necrophorum*, respectively. All the 250 samples were screened individually for both the organisms by LAMP. Among them, 104 (41.6%) were found to be positive for *D. nodosus* and 120 (48.0%) were positive for *F. necrophorum*. The efficacy of LAMP in terms of sample DNA detection limit was compared with the PCR by using standard dilutions of DNA extracted from *D. nodosus* and *F. necrophorum* cultures. The detection limit was found to be higher than PCR for both the organisms. The sensitivity of LAMP is compared with PCR by targeting 16S rRNA gene of *D. nodosus* and *lktA* gene of *F. necrophorum*. In case of *D. nodosus*, out of 250 samples, 75 (30.0%) were positive by PCR and 104 (41.6%) were positive by LAMP. Among 250 samples, 85 (34.0%) were positive by PCR and 120 (48.0%) were positive by LAMP in case of *F. necrophorum*. The LAMP was found to be more sensitive than PCR in detecting the organisms with high statistical significance.

**Keywords** Footrot · *D. nodosus* · *F. necrophorum* · LAMP · Duplex PCR · Sheep and goat

**Introduction**

Livestock is the major contributing factor for India being a developing country which mainly depends on agriculture as the main source of income. Sheep and goats play an important role in the livelihood of many small and marginal farmers and landless laborers. Footrot has been reported previously from temperate climates of Jammu and Kashmir alone for the last 18 years. In India, the disease was reported later on from unusual tropical climate of Andhra Pradesh and Tamil Nadu states of southern India. Outbreaks of footrot were being reported regularly from Andhra Pradesh affecting sheep and goat population (Wani et al. 2007; Farooq et al. 2010; Thomas et al. 2011; Sreenivasulu et al. 2013; Kumar et al. 2013a, b, 2015, 2016a, b). At present, footrot is endemic disease in the states of Jammu and Kashmir, Uttar Pradesh, Himachal Pradesh, and Andhra Pradesh.

Footrot is mainly caused by the synergistic action of *Dichelobacter nodosus* formerly (*Bacteroides nodosus*) as the primary transmitting agent and *Fusobacterium necrophorum* as secondary pathogen. Both are anaerobic, gram-negative, rod shaped bacterium with characteristic knobs at each end and are fimbriated (Sreenivasulu et al. 2013). Clinically severe disease is referred as the virulent footrot, while milder mainly interdigital form of the disease refers to benign footrot, based on the production of thermostable proteases or the integrase A gene (*intA*), which relate to the corresponding forms of the disease (Cheetham et al. 2006). The term intermediate footrot is used to refer forms of footrot that are in between the two forms. The organism *Dichelobacter nodosus* was classified as 10 serogroups (A–I and M) identified based on K-type agglutination of the surface antigens. These serogroups are further divided into 19 serotypes based on cross-absorption tests. Type IV fimbriae and extracellular proteases are essential for virulence
of *D. nodosus* (Kennan et al. 2001, 2010). Class I fimbriae included those of serogroups A, B, C, E, F, G, and I, while class II contained in serogroups D and H. The two sets of fimbriae are also distinguished by their disulfide loop profile (Elleman 1988).

*Fusobacterium necrophorum* subsp. *necrophorum* produces a secreted protein leukotoxin (*lktA*) a major virulence factor along with other potent virulence factors such as leukotoxin, lipopolysaccharide, and hemagglutinin (Tan et al. 1992, 1996; Amoako et al. 1993; Narayanan et al. 2001; Kumar et al. 2013a, b).

Diagnosis of footrot disease by isolation of the organism, followed by antigenic analysis using serological procedures, may take 3–4 weeks. Alternatively PCR- and qPCR-based methods without need to culture have been used for initial detection (La Fontaine et al. 1993; Liu and Webber 1995; Dhungyel et al. 2002; Cheetham et al. 2006; Belloy et al. 2007; Frosth et al. 2012) and characterization (Dhungyel et al. 2002) of *D. nodosus*. Nucleic acid amplification by PCR is one of the most valuable alternatives for the detection of *D. nodosus* causing footrot. High precision instruments and standardized protocols are essential for PCR-based methods for detection and confirmation of the amplified products. In addition, PCR is not suitable to adopt for routine clinical use. The loop-mediated isothermal amplification (LAMP) assay may fulfill all the above parameters for which it can be used as low-cost alternative for detection of organisms causing footrot.

**Materials and methods**

**Collection of samples**

A total of 250 foot swabs were collected from the flocks of sheep and goats in different districts of Rayalaseema regions (Chittoor, Kadapa, Nellore, Anantapur) where the prevalence of footrot is high (Table 1). Among them, 200 were collected from sheep and 50 are from goats. Samples were collected from the animals showing the symptoms like lameness, decreased body weight, infectious dermatitis of the interdigital skin with a gray scum, pungent, and characteristic rotting smell.

| S. no. | Name of the district | No. of flocks visited | No. of samples collected | Sheep | Goats | Total |
|-------|----------------------|-----------------------|--------------------------|-------|-------|-------|
| 1     | Chittoor             | 40                    | 107                      | 15    | 122   |
| 2     | Kadapa               | 15                    | 38                       | 9     | 47    |
| 3     | Nellore              | 6                     | 35                       | 12    | 47    |
| 4     | Anantapur            | 5                     | 20                       | 14    | 34    |
|       | Total                | 66                    | 200                      | 50    | 250   |

**Reference strain**

The *F. necrophorum* DNA was provided by University of Warwick, Warwickshire, England, UK with Ref no: Fn DSM 21784. *D. nodosus* DNA was prepared from stock cultures (serogroup I) maintained in this laboratory.

**Screening of clinical samples by duplex PCR**

DNA was extracted by boiling method and all the samples were screened by duplex PCR for the presence of the 16S rRNA of *D. nodosus* and *lktA* gene of *F. necrophorum*. The duplex PCR was performed using a thermocycler with a final reaction volume of 25 μL containing 2.5 μL of Taq buffer (10×); 1 μL of 25 mM MgCl2; 0.3 μL of d NTP mix (10 mM); 0.6 μL of 16S rRNA (F+R) (10 pmol); 0.6 μL of *lktA* (F+R) (10 pmol); 0.5 μL of Taq DNA polymerase (5 U/μL from Invitrogen, Thermo Fisher Scientific); 3.0 μL of template DNA; and 16.5 μL of nuclelease-free water. Amplification was obtained with 35 cycles, following the PCR 10 μL of amplified products was confirmed by using gel electrophoresis in a 1.5% w/v agarose gel. The amplified bands were visualized under UV illumination.

**Primer designing for LAMP**

The LAMP primers targeting *D. nodosus* 16S rRNA and *lktA* gene of *F. necrophorum* were designed by using the LAMP primer design software program Primer Explorer V5, from Ekin Chemical Company, Japan (http://primerexplorer.jp/elamp3.0/index.html). A set of four AT rich primers comprising two outer and two inner primers were designed. The two outer primers were known as the forward outer primer (F3) and the backward outer primer (B3) which helps in strand displacement. The inner primers were known as the forward inner primer (FIP) and the backward inner primer...
(BIP), respectively. FIP contains F1C (complementary to F1), a TTTT spacer, and the F2 sequence. BIP contains the B1C sequence (complementary to B1), a TTTT spacer, and B2 sequence (Tables 2 and 3). The primers were procured from Eurofins Genomics India Pvt. Ltd., Bangalore.

**Standardization of LAMP reaction**

LAMP reaction was standardized for 16S rRNA of *D. nodosus* and lktA gene of *F. necrophorum*. The enzyme Bsm DNA polymerase was procured from Thermo Scientific, Inc. The LAMP was standardized to determine optimum concentrations of primers, enzyme, temperature, and time combinations for amplification of target genes. For *D. nodosus*, a total volume made up to 25 μL using nuclelease-free water with MgCl2 (25 mM)—1 μL; Bsm buffer (×10)—2.5 μL; dNTP mix (10 mM)—7.5 μL; Bsm DNA polymerase (8 U/μL)—0.5 μL; F3 (10 pmol/μL)—1 μL; B3 (10 pmol/μL)—1 μL; FIPα (40 pmol/μL)—4 μL; BIP (40 pmol/μL)—4 μL; and nuclease-free water—2.2 μL. In case of *F. necrophorum*, a reaction mixture of 25 μL was prepared with 2.0% w/v agarose using 1xTBE buffer pH 8.0 (Tris base 54 g, boric acid 27.5 g, and 20 mL of 0.5 M EDTA). The LAMP products were mixed with 2 μL of 6× Loading buffer and subjected to agarose gel electrophoresis. The LAMP amplification was done in thermal cycler by the following steps with negative controls added with nuclelease-free water in place of template DNA. In the initial reaction, nuclelease-free water, Bsm buffer (×10), dNTPs mix, outer primers, inner primers, enzyme, and template were added into Eppendorf tube. For *D. nodosus*, LAMP reaction was standardized at 60 °C for 60 min as shown in Fig. 1 followed by enzyme inactivation step for 10 min at 80 °C. In case of *F. necrophorum*, the LAMP reaction was standardized at 56 °C for 60 min as shown in Fig. 2. The amplified LAMP products were stored at −20 °C.

**Electrophoresis of LAMP products**

LAMP products were subjected to agarose gel electrophoresis in a Genei submerged gel apparatus. Agarose gels were prepared with 2.0% w/v agarose using 1xTBE buffer pH 8.0 (Tris base 54 g, boric acid 27.5 g, and 20 mL of 0.5 M EDTA). The LAMP products were mixed with 2 μL of 6× Loading buffer and subjected to agarose gel electrophoresis. The LAMP amplification was done in thermal cycler by the following steps with negative controls added with nuclelease-free water in place of template DNA. In the initial reaction, nuclelease-free water, Bsm buffer (×10), dNTPs mix, outer primers, inner primers, enzyme, and template were added into Eppendorf tube. For *D. nodosus*, LAMP reaction was standardized at 60 °C for 60 min as shown in Fig. 1 followed by enzyme inactivation step for 10 min at 80 °C. In case of *F. necrophorum*, the LAMP reaction was standardized at 56 °C for 60 min as shown in Fig. 2. The amplified LAMP products were stored at −20 °C.

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**LAMP amplification**

The LAMP amplification was done in thermal cycler by the following steps with negative controls added with nuclelease-free water in place of template DNA. In the initial reaction, nuclelease-free water, Bsm buffer (×10), dNTPs mix, outer primers, inner primers, enzyme, and template were added into Eppendorf tube. For *D. nodosus*, LAMP reaction was standardized at 60 °C for 60 min as shown in Fig. 1 followed by enzyme inactivation step for 10 min at 80 °C. In case of *F. necrophorum*, the LAMP reaction was standardized at 56 °C for 60 min as shown in Fig. 2. The amplified LAMP products were stored at −20 °C.

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gel loading dye and 10 μL of the products was mixed and loaded in each well. Electrophoresis was carried 80 V for 45 min at 35 mA. Electrophoresis was stopped when the dye front reached two-third of the gel. Gel was visualized under ultraviolet (UV) transilluminator and photographed with gel documentation system (Alpha Innotech, Alphaimager, HP).

**Determination of specificity of F. necrophorum and D. nodosus LAMP**

In specificity assay, cross-amplification of *F. necrophorum* LAMP was tested using the DNA extracted from the pure cultures of *E. coli*, *S. aureus*, and *D. nodosus*. Similarly, specificity of *D. nodosus* LAMP was tested using *E. coli*, *S. aureus*, and *F. necrophorum* DNA.

**Comparison of efficacy of LAMP with PCR**

The efficacy of LAMP in terms of sample DNA detection limit was compared with the PCR using the standard doubling dilutions of *D. nodosus* culture DNA and *F. necrophorum* DNA provided by University of Warwick, Warwickshire, England, with Ref no: Fn DSM 21784. The dilutions were performed with initial ten-fold followed by two-fold dilutions.

**Comparison of sensitivity of PCR and LAMP**

All the 250 field samples were tested with LAMP and the results were compared with duplex PCR results. The diagnostic sensitivity of the LAMP was compared with duplex PCR results using chi-square analysis.

**Screening of clinical samples by both PCR and LAMP**

All the 250 clinical samples were screened by both duplex PCR and LAMP as per standard protocol.

**Results and discussion**

Footrot is a rapid spreading contagious disease affecting productivity of sheep and goats. Conventional, clinical diagnosis is based on visual inspections, with occasional laboratory diagnosis for identification of causative agent. The available laboratory and molecular methods like PCR for diagnosis are cumbersome and require specialist growth media and expertise. Alternatively, a novel nucleic acid amplification LAMP by autocycling strand displacement DNA synthesis using *Bst* DNA polymerase large fragment was first designed by Notomi et al. (2000) and Mori et al. (2001). Subsequently, LAMP assays have been developed for several livestock diseases (Thekisoe et al. 2005; Dukes et al. 2006; Alhassan et al. 2007; Kaneko et al. 2007; Sun et al. 2011; Das et al. 2012; Radhika et al. 2016; Best et al. 2018) and are consistently proven to be tolerant to common biological inhibitors of conventional PCR assays, from serum, plasma, urine, and feces LAMP reported to offer a field suitable, easily performed molecular diagnostic assay for detection of early infection.

The outer LAMP primers could anneal to the complementary sequence of the double standard DNA in dynamic equilibrium in LAMP reaction mixture at the temperature around 65 °C to initiate LAMP reaction. Initial denaturation step of template DNA was widely applied to increase the reaction efficiency (Notomi et al. 2000; Kamachi et al. 2006; Das et al. 2012; Radhika et al. 2016; Best et al. 2018). However, Parida et al. (2008) recommended that initial heat denaturation step is not required in LAMP reaction. Accordingly, the LAMP reaction was performed without initial heat denaturation step in the present study.

For standardization of LAMP, different combinations of specific outer and inner primers were tried. Serial dilutions of primers with 10 pmol/μL of outer primer and the inner primers of 10 pmol/μL, 40 pmol/μL, and 80 pmol/μL were
tested. The outer primer concentration of 10 pmol/μL and 40 pmol/μL of inner primers (FIP and BIP) were found to be optimum for amplification of specific positive LAMP reaction for *D. nodosus* and *F. necrophorum*. Notomi et al. (2000), Radhika et al. (2016), and Best et al. (2018) recommended similar combination of outer primers at 1/4 to 1/10 concentration of the inner primers for optimal amplification. The enzyme *Bsm* polymerase large fragment with high functional similarity to *Bst* DNA polymerase was used successfully in the present study, as described by Johnson et al. (2014) to amplify Citrus yellow mosaic badnavirus (CMBV) by LAMP. The LAMP reaction was standardized by using different concentrations of the enzyme 0.3 μL, 0.5 μL, 0.75 μL, and 1 μL to test the optimum concentration. The enzyme concentration of 0.3 μL was found to be optimum for positive LAMP reaction. Similarly, 0.5 μL enzyme concentration was found to be optimum for LAMP reaction to detect *F. necrophorum* from footrot suspected sheep and goat samples. LAMP reaction was standardized at 60 °C for 60 min for *D. nodosus*. In case of *F. necrophorum*, the LAMP reaction was standardized at 56 °C for 60 min. Similar combinations of temperature and time ranging from 60 to 65 °C have been widely applied in previous studies for successful LAMP reaction with *Bst* DNA polymerase (Notomi et al. 2000; Radhika et al. 2016; Zheney et al. 2018; Best et al. 2018; Zhang et al. 2013; Wang et al. 2020).

The specificity of LAMP for *F. necrophorum* was found to be 100% showing no cross-amplification with the DNA extracted from the pure cultures of *E. coli*, *S. aureus*, and *D. nodosus*. Similarly, none of the samples tested using *E. coli*, *S. aureus*, and *F. necrophorum* DNA extracts revealed no cross-amplification for *D. nodosus* LAMP showing 100% specificity. Best et al. (2019) reported decrease sensitivity of LAMP with dirty and dry foot swabs against the clean and moist swabs from the affected cases. In the present study, the clean foot swabs were collected without dirt and feces and dipped the swabs in saline tubes immediately at the site of collection and transported to the lab to avoid drying.

The efficacy of LAMP in terms of sample DNA detection limit was compared with the PCR by using standard dilutions of DNA extracted from *D. nodosus* cultures after measuring the DNA concentration with biospectrometer (Eppendorf). The PCR could detect up to the dilution of 1:320 (0.7 ng/μL), whereas the LAMP could detect up to the dilution of 1:1280 (0.2 ng/μL) as presented in Fig. 3. The DNA detection limit of LAMP was found to be much higher than PCR. Similarly, the LAMP for *F. necrophorum* could detect up to 1:1280 (0.0125 ng/μL) of DNA dilution, whereas for PCR could detect only up to 1:320 (0.05 ng/μL) dilution as shown in Fig. 4. Sun et al. (2011) and Saxena et al. (2019) performed similar type of sensitivity assay of LAMP by tenfold dilutions of genomic DNA copies of target species. Best et al. (2018, 2019) designed VDNLAMP to detect virulent and benign *D. nodosus* from ovine foot suitable for in-field use in Australian conditions with wide variations in ambient temperature. They observed increased sensitivity of the test in the laboratory with a decrease in Ct values of the aprV2/aprB2 rtPCR and recorded 89% sensitivity and 97% specificity of the VDN LAMP in comparisons with observed clinical signs of footrot in flocks. In the present report, the
LAMP was designed to detect both *D. nodosus* and *F. necrophorum* from footrot cases of sheep and goats suitable for in-field use in Indian conditions without wide ambient temperature variations. The test could perform satisfactorily in lab with 250 samples and the results were compared with that of duplex PCR to determine the diagnostic sensitivity of the LAMP test (Table 4). Out of 250 samples tested, 75 were positive for *D. nodosus* by PCR and 104 were positive for LAMP. The chi-square calculated value was found to be 7.318. Similarly, for *F. necrophorum*, out of 250 samples 85 were positive by PCR and 120 were positive for LAMP (Fig. 5). The chi-square calculated value was 10.128. The diagnostic sensitivity of LAMP for *D. nodosus* and *F. necrophorum* was found to be significantly high compared with conventional duplex PCR designed in this lab for simultaneous detection of both the organisms.

**Conclusion**

LAMP technology is still in the early stages of full pledged field application. Further understanding of contributing factors that impact performance, the machinations of use in-field and further validation of the present LAMP kit for early detection of footrot got major advantages. It could be performed by non-specialist with minimal training at the field level which can avoid difficult and time-consuming procedures. Screening of the samples with LAMP for both *D. nodosus* and *F. necrophorum* will help in epidemiological investigation to establish the causal association of *F. necrophorum* with *D. nodosus* in causing footrot in sheep and goats. It will also facilitate early, fast, and accurate on-farm diagnosis of infection and help to reduce spread of the disease through adoption of suitable control and preventive measures including vaccination.

**Author contribution** VK conceived and designed research. MK collected the samples and conducted experiments. VK analyzed data. MK wrote the manuscript. SB and VK read corrected and approved the manuscript.

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**Data availability** Not applicable.

**Code availability** Not applicable.

**Declarations**

**Ethics approval** No animal ethical issues are involved in the current research.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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