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Full Length Research Paper

Synergistic LAMP assays for identifying hoof swabs in dairy cattle affected by footrot

Sun D. B., Wu R., Guo D. H.*, He X. J., Zheng J. S., Wang J. F., Lin Y. C., Han X., Wang Y. Q. and Guo T. T.

College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing High-tech Industrial Development Zone, Daqing 163319, P. R. China.

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To investigate the presence of *Fusobacterium necrophorum* (*F. necrophorum*) and *Dichelobacter nodosus* (*D. nodosus*) in dairy cattle footrot, a loop -mediated isothermal amplification method for detection of *D. nodosus* (Dn-LAMP) was developed and evaluated using species-specific16S rRNA gene as target gene. The Dn-LAMP showed no cross reaction with several common pathogens from cattle, and detection limit of the Dn-LAMP was 10 cfu/mL of *D. nodosus*. Amplicons of the Dn-LAMP could be detected by visual inspection. 189 hoof swabs from footrot-infected dairy cows in Heilongjiang province of China were detected using Dn -LAMP and Fn-LAMP for diagnosis of *F. necrophorum*, respectively. In 189 hoof swabs, 9 were positive for *D. nodosus*, and 95 were positive for *F. necrophorum*. These data suggested that combination of Dn -LAMP with Fn-LAMP will have a potential use for etiology investigation of footrot in dairy cattle.

Key words: *Dichelobacter nodosus*, *Fusobacterium necrophorum*, LAMP.

INTRODUCTION

Cattle footrot is a highly contagious disease characterized by lameness, loss of body condition, poor wool production, and susceptibility to other infectious diseases (Adams et al., 1960; Smedgaqrd et al., 1963). The disease has frequently broken out in many cattle-raising countries and remains a severe economic loss due to additional labor, antibiotics, feed, and drug residues in individual ranchers (Gurung et al., 2006; Gilder et al., 1960; Vladutilu et al., 1971; Li et al., 2000). In original study, *Fusobacterium necrophorum* (*F. necrophorum*) and *Bacteroides melaninogenicus* are identified as etilogical agents of cattle footrot (Berg and Loan, 1975).

However, *Dichelobacter nodosus* still exists in hoof in footrot cattle from a recent report (Bennett et al., 2009). But little in subsequent study, *D. nodosus* is isolated and identified in foot lesions from cattle (Richards et al., 1980). At present, study on cattle footrot mainly focuses on leukotoxin of *F. necrophorum* which is a major virulence factor and capable of eliciting formation of immune protection against *F. necrophorum* infection (Narayanan et al., 2003; Sun et al., 2009; Guo et al., 2010; Narayanan et al., 2002). Information can be obtained regarding etiology investigation of *D. nodosus* and *F. necrophorum* in cattle footrot, especially in China. Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification-based diagnostic technique under isothermal condition, and is widely used for diagnosis of pathogen (Notomi et al., 2000; Sun et al., 2010a). The purpose of our study is to develop the LAMP method (Dn-LAMP) for rapid detection of *D. nodosus*, and investigate the presence of *D. nodosus* and *F. necrophorum* in hoof swabs of dairy cattle footrot in Heilongjiang province of China by combination of the Dn-LAMP method with the Fn-LAMP method (Sun et al., 2010b).

*Corresponding author. E-mail: amu0406@yahoo.com.cn. Tel: +86-459-6819200. Fax: +86-459-6819190.

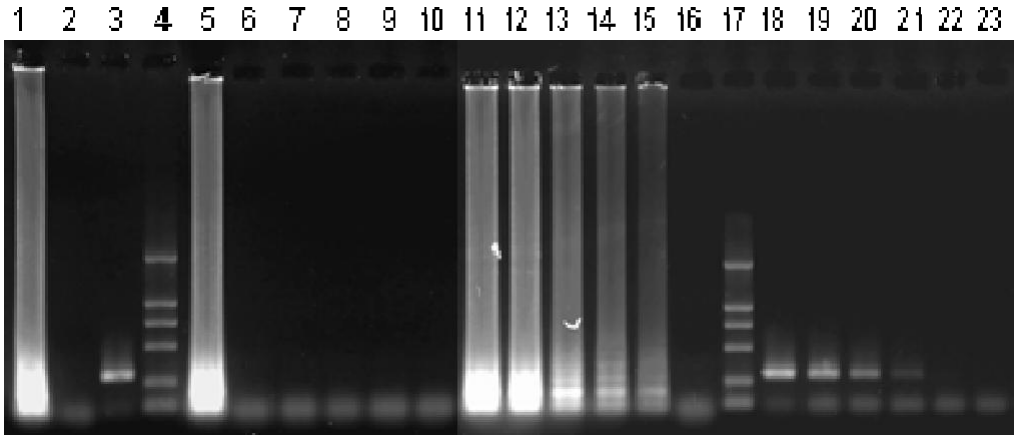

Figure 1. Dn-LAMP and PCR amplification, Dn-LAMP specificity assay, and Dn-LAMP sensitivity assay. Lane 1-4: Dn-LAMP and PCR amplification. Lane 1: Dn-LAMP products, Lane 2: Dn-LAMP negative control, Lane 3: PCR products, Lane 4: DNA Marker DL2000. Lane 5-10: Specificity assay of Dn-LAMP. Lane 5: Dn-LAMP positive control, Lane 6: *F. necrophorum*. Lane 7: enterotoxigenic *Escherichia coli*, Lane 8: *Salmonella*, Lane 9: *Pasteurella multocida*, Lane 10: Dn-LAMP negative control. Lane 11-23: Sensitivity assay of Dn-LAMP. Lane 11- 16: Dn-LAMP products of *D. nodosus* from 10^5 cfu/mL to 1 cfu/mL, respectively, Lane 17: DNA Marker DL2000. Lane 18-23: PCR products of *D. nodosus* from 10^5 cfu/mL to 1 cfu/mL, respectively.

MATERIALS AND METHODS

Bacterial strains

The virulent strain type of *D. nodosus* strain 198A (VPI 5731-1, ATCC 27521), was obtained from American Tissue Culture Collection (ATCC, Rockville, MD, USA). *F. necrophorum* strain H05 (Genbank accession no. DQ672338) and other control strains were field isolates, and were stocked in Clinical Veterinary Medicine Laboratory, College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University.

Primers for Dn-LAMP and PCR

A total of four oligonucleotide primers for Dn-LAMP were designed according to species-specific16S rRNA gene of *D. nodosus* (Genbank accession no. M35016) using PrimerExploer V3 software online (http://primerexplorer.jp/e/). Primer sequences were as follows: Dn-F3: 5'CGTGGGTAGCAAACAGGATT3', Dn-B3: 5'TCTTCGCGTTGCATCGAATT', Dn-FIP: 5'TGCGTTAGCTTCGGTACCGAGTCTGGTAGTCCACGCCCTA3', Dn-BIP: 5'TAAGTTGACCGCCTGGGGAGTCATGCTCCACCGCTTGTG3'. To verify Dn-LAMP, the primers Dn-F: 5' TAGTGGCGGACGGGTGAG3', and Dn-R: 5'GGCATTGCTGGATCAGGG3' were designed for developing the conventional PCR of *D. nodosus* according to species-specific16S rRNA gene.

Establishment of Dn-LAMP

After determining conventional reaction conditions, the Dn-LAMP method was evaluated by specificity assay, sensitivity assay, and visible detection of amplicons. In specificity assay, cross-reactions of Dn-LAMP with other pathogens were tested using genomic DNA from common pathogens of cattle as templates. In sensitivity assay, detection limit of Dn-LAMP was determined using genomic DNA from ten-fold serial dilutions of *D. nodosus* strain 198A as templates, and comparison of Dn-LAMP with conventional PCR was carried out under the same conditions. The visible detection of Dn-LAMP amplicons was performed through direct naked-eye observation and fluorescent dyes observation using EvaGreen™ as reagent under UV-illumination.

Detection of clinical samples

189 foot swabs from footrot-infected dairy cows were collected from 5 farms in Heilongjiang province, Northeast China. All foot swab specimens were dissolved in 1 ml of phosphate buffered saline (PBS, pH 7.4). The genomic DNA of each sample was extracted using BacteraGen DNA Kit (CWBIO, Beijing, China) according to manufacturer's protocol, respectively. Ten microliter extracted genomic DNA from each foot swabs was used for detection of *D. nodosus* and *F. necrophorum* by Dn-LAMP and Fn-LAMP.

RESULTS AND DISCUSSION

Results of Dn-LAMP and PCR amplification, Dn-LAMP specificity assay, and Dn-LAMP sensitivity assay are shown in Figure 1. The agarose gel electrophoresis showed that a clear ladder-like DNA band was observed in the lane of Dn-LAMP products, and approximately 300 bp of DNA band was observed in the lane of PCR products. The detection limit of Dn-LAMP is 10 cfu/mL of *D. nodosus*, and was 10 times more sensitive than that of the conventional PCR. Dn-LAMP had no cross reactions with genomic DNAs from *F. necrophorum*, enterotoxigenic *Escherichia coli*, Salmonella and *Pasteurella multocida*. Result of the visible detection of

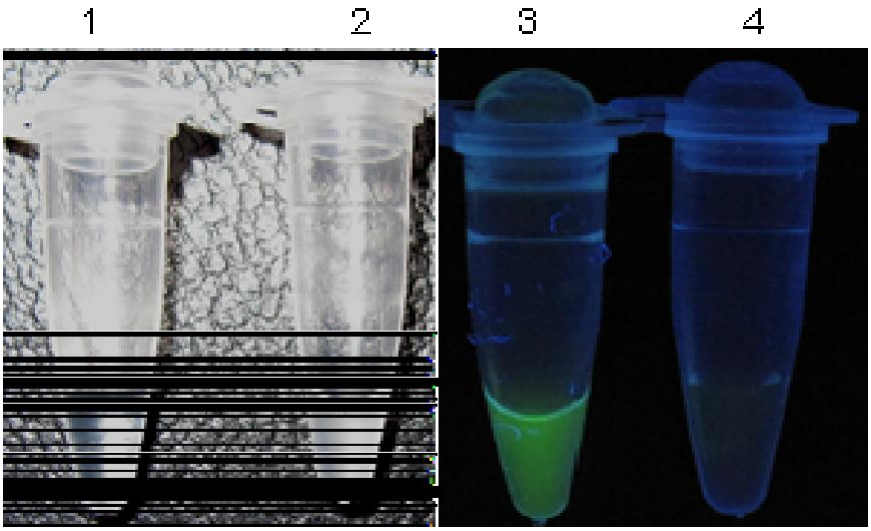

Figure 2. Visible detection of Dn-LAMP products. 1. Negative reaction tube of Dn- LAMP by the direct naked-eye observation. 2. Positive reaction tube of Dn-LAMP by the direct naked-eye observation. 3. Positive reaction tube of Dn-LAMP by the fluorescent dyes observation. 4. Negative reaction tube of Dn-LAMP by the fluorescent dyes observation.

Table 1. Detection of clinical samples by Dn-LAMP and Fn-LAMP.

Farms	Samples	*D. nodosus*	*F. necrophorum*
A	33	0	11
B	48	3	27
C	55	2	32
D	42	3	19
E	11	1	6
Total	189	9	95
Percentage		4.8%	50.3%

Dn-LAMP amplicons is shown in Figure 2. The direct naked-eye observation indicated that positive reaction tube of Dn-LAMP appeared to be flocculent white precipitate, and negative reaction tube of Dn- LAMP appeared to be clear solution. The fluorescent dyes observation demonstrated that positive reaction tube of Dn-LAMP appeared to be strong green fluorescence, and negative reaction tube of Dn-LAMP appeared to be weak or no green fluorescence. Result of detection of clinical samples revealed that in 189 foot swabs from footrot-infected dairy, 9 were positive for *D. nodosus* (4.8%), 95 were positive for *F. necrophorum* (50.3%) (Table 1).

D. nodosus isolates are divided into nine different serogroups based on their fimbrial antigens (Claxton et al., 1983). The species-specific 16S rRNA gene is highly conserved in the different serogroups of *D. nodosus* isolates. The species- specific 16S rRNA gene as a target gene has been widely applied in the nucleotide diagnostic approach of *D. nodosus* (La Fontaine et al., 1993). In this study, a loop-mediated isothermal amplification method (Dn-LAMP) was developed for detection of *D. nodosus* using four specific primers targeting the conserved species-specific 16S rRNA gene. Compared with conventional PCR method, the most advantages of Dn-LAMP are that Dn-LAMP can be performed in the thermal water bath, and its amplicons could be determined by direct visual observation or fluorescent dyes observation instead of the electrophoresis with ethidium bromide contamination. In detection of clinical samples, combination of Dn-LAMP with Fn-LAMP demonstrates greater availability in survey of footrot pathogen. A total of 189 foot swabs from footrot-infected dairy cows from 5 farms in Heilongjiang province, northeast China were tested for analyzing the presence of *D. nodosus* and *F. necrophorum* using Dn-LAMP and Fn- LAMP. 9 were positive for D. nodosus, and 95 were positive for *F. necrophorum*. Test result of the limited samples suggests that combination of Dn-LAMP with Fn-LAMP will have a potential use for confirming the presence of *D. nodosus* and *F. necrophorum* in footrot of cattle. Meanwhile *D. nodosus* should be highly concerned when controlling cattle footrot in China.

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