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Comparison of culture, ELISA and LAMP-PCR for diagnosis of *Mycobacterium avium subsp. Paratuberculosis*

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Abstract

Mycobacterium avium subsp. Paratuberculosis is the etiologic agent of Johne's disease in the cattle. JD is a disease with considerable economic impact on dairy cattle. It is prevalent worldwide, especially in developing countries including Iran. Rapid and accurate diagnosis of the infection is essential for controlling the disease. The present study compared fecal culture, ELISA and loop-mediated isothermal amplification (LAMP) for the diagnosis of MAP in dairy cattle. A total of 225 serum and fecal samples were collected from 14 dairy cattle farms in Tehran, Iran. The fecal samples were cultured in Harrold's egg yolk agar with and without mycobactin J. DNA was extracted directly from fecal samples, too. Serum ELISA and LAMP were compared with fecal culture in terms of sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratio of a positive test result, and likelihood ratio of a negative test result. The sensitivity of LAMP and ELISA was 100% and 79%, respectively. Their specificity was 83.33% and 93%, respectively. Both LAMP ($P < 0.001$; $k = 0.167$) and ELISA ($P < 0.001$; $k = 0.374$) were in agreement with fecal culture as the gold standard. Moreover, LAMP and ELISA had 80% agreement with each other. This study suggests LAMP as a valuable and cost-effective tool for the early diagnosis of JD caused by MAP. Furthermore, due to inability of ELISA in detecting early stages of the disease and long and expensive method of culture, LAMP could be used alongside ELISA in cattle-wide screening for disease.

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Introduction

Mycobacterium avium subsp. paratuberculosis (MAP) is the etiologic agent of paratuberculosis (PTB) or Johne's disease (JD). JD, chronic, debilitating enteritis in ruminants, has a worldwide distribution and imposes a significant global economic impact. Affected animals have chronic, sometimes intermittent, diarrhea and progressive emaciation. A possible link between MAP and Crohn's disease, a human inflammatory bowel disease, has been suggested. MAP infections are largely subclinical and characterized by a prolonged incubation period, roughly five years in dairy cattle (Whitlock *et al*,. 2010).Their diagnosis is generally challenging due to the pathogen's fastidious nature (and the resultant complex in-vitro growth requirements) and low-level intermittent fecal shedding during the preclinical phase of the infection. Detection of these low-shedders is critical to effective JD control as these animals serve as sources of infection for susceptible calves (Salgado *et al*,. 2013) Various methods such as culture, immunological tests, and histopathological assessment of lesions have been proposed to diagnose latently infected animals. However, the slow-growing nature of MAP makes the definitive diagnosis of subclinical infections difficult. While conventional culturing is commonly regarded as the gold standard for JD diagnosis in cattle (Clark *et al*,. 2008), the method is known to have several disadvantages including long incubation time (up to 16 weeks), high labor intensity, and the probability of false negative results in subclinical animals (Collins,. 1996) Moreover, culture methods require six months to confirm that a sample is negative for MAP (Carvalho *et al*,. 2012). Enzyme-linked immunosorbent assay (ELISA) can be rapidly perform, but its sensitivity is limited by the fact that the antibodies may not be detectable until late infection. Polymerase chain reaction (PCR) has been widely applied to improve the identification of microorganisms and overcome the shortcomings of traditional microbiological detection methods. However, several factors such as the excessive nonspecific DNA derived from the host or other microbes, the quality of the genomic DNA preparation, and the presence of substances that inhibit PCR amplification in clinical samples may affect the performance of PCR. In fact, large amounts of irrelevant genomic materials and high concentrations of inhibitors (e.g. bile salts, bilirubin, urobilinogen, and polysaccharides) in fecal samples make them inappropriate candidates for PCR. Furthermore, the necessity of electrophoresis to detect the amplified products turns the process into a time-consuming task (Khare *et al*,. 2004). Loop-mediated isothermal amplification (LAMP) has thus been recently suggested as a rapid technique for DNA amplification (Mori and Notomi,. 2009). LAMP employs a minimum of four specially designed primers, i.e. a forward outer primer (F3), a backward outer primer (B3), a forward inner primer (FIP) comprising two binding domains (F1c and F2), and a backward inner primer (BIP) consisting of two binding domains (B1 and B2c). The combination of these primers facilitates the recognition of six specific regions within the target genetic locus. Additional primers including a forward loop primer (FLP) and a backward loop primer (BLP) are typically optional and may be used to accelerate or enhance the sensitivity of the LAMP assay (Notomi *et al*,. 2000). Predictably, LAMP assays tend to have high specificity, as the amplification occurs only when six or eight specific regions of the target amplicon are recognized by the primers. Moreover, while bile salts, bilirubin, urobilinogens, and polysaccharides have been previously reported to inhibit *Taq* DNA polymerase used in PCR(Khare *et al*,. 2004) , *Bst* DNA polymerase used in LAMP has been presumed to be unaffected(Melville *et al*,. 2014). In addition, despite its high accuracy, LAMP assay does not require expensive equipment and too many steps (as is the case with conventional PCR). Due to the high amplification efficiency of LAMP, up to 10^9 copies of a target can be accumulated in less than one hour of incubation (Saleh *et al*,. 2008). The LAMP assay may be monitored by measuring the turbidity of magnesium pyrophosphate (a by-product of LAMP),

measuring fluorescence using a DNA intercalating dye such as SYBR® Green or a metal ion-binding fluorophore such as calcein, and/or by color change using a metal ion-binding indicator dye and agarose gel electrophoresis (Yang *et al*,.2010; Parida *et al*,. 2008). Considering these advantages, LAMP has become a valuable tool for the rapid diagnosis of infectious diseases.

The present study was carried out to compare Herrold's egg yolk agar (HEYA) culture as the gold standard, LAMP and serum ELISA methods for the diagnosis of *Mycobacterium avium subsp. Paratuberculosis* in dairy cattle.

Material and methods

Bacterial strains

Bacterial strains used to standardize the LAMP method in the present study were MAP 316F provided by the Tuberculosis Department of Razi Vaccine and Serum Research Institute of Tehran, Iran. Additional strains including *Mycobacterium bovis AN5, Mycobacterium tuberculosis DT,* and *Mycobacterium avium subsp. avium* were also used to determine the LAMP specificity.

Sample collection

A total of 225 serum and fecal samples were collected from 14 dairy cattle farms in Tehran Province, Iran. The fecal samples were directly taken from the cows' rectum. Samples were immediately placed in a container with ice packs and transported to the tuberculosis laboratory of Razi Vaccine and Serum Research Institute (Tehran, Iran). The fecal samples were then stored at -70°C until bacterial culture. Serum samples were maintained at -20°C until preparation for ELISA.

Culture of fecal samples

Each fecal sample was used for two procedures, i.e. decontamination processing as well as DNA extraction and LAMP. The samples were decontaminated, homogenized, and cultured on HEYM with and without mycobactin J at 37°C for 8-16 weeks (OIE,. 2014). The samples in which the bacteria could merely grow on HEYM containing mycobactin J were considered positive. Additional tests such as colony morphology, acid-fast staining, and PCR-IS900 assay were performed to confirm the isolated bacteria.

DNA extraction from culture and feces

MAP DNA was extracted from the culture according to the method described previously by Van Soolingen *et al.* (Van Soolingen,. 2001). DNA was also directly extracted from all fecal samples. DNAs were extracted from bout 100-200 mg of fecal samples using a stool-DNA extraction kit (AccuPrep, Bioneer Corp., Korea) according to the manufacturer's instructions. The samples were eluted with 50 µl of the kit-supplied elution buffer.

LAMP

Primer Explorer V3 (htpp://primerexplorer.jp/ elamp3.0.0/index.html), a LAMP primer designing software package, was used to design four primers including two outer primers (F3 and B3) and two inner primers (FIP and BIP). Two loop primers (LF and LB) were manually designed based on the insertion sequence of IS900 obtained from the GenBank (Accession number: AF416985) (Chamberlin *et al*,. 2001; Bull *et al*,. 2000). Nucleotide sequences of the functional LAMP primers are shown in Table 1. The LAMP assays were carried out in 25 µl of final reaction mixture containing 60 pM of each F3, B3, FIP, and BIP, 30 pM of each FLP and BLP, 7.5 mM $MgSO_4$, 40 mM dNTP, 0.8 M betaine (Sigma-Aldrich Corp., USA), 8 U Bst DNA polymerase (New England Biolabs, USA), 1 µl nuclease-free water, and 2.5 µl DNA sample. The LAMP reaction was performed in a thermal mixer. For comparison purposes, the reaction was also conducted using a conventional thermal cycler which showed both machines to work well. To find the optimum time and temperature, the reactions were performed at 58-68°C for 15-120 minutes. Finally,

each reaction was incubated at 63°C for 60 minutes. A positive and negative control was included in each run. The LAMP products were directly detected with the naked eye through the formation of white precipitations and adding 2 µl of SYBR® Green I (Sigma-Aldrich Corp., USA) to the reaction tube and observing the color of the solution under an ultraviolet (UV) transilluminator. Furthermore, microtube centrifugations were also carried out to help the detection of primary turbidity by the formation of precipitation. In addition, LAMP products were electrophoresed on 2% agarose gel with red gel staining and evaluated in a UV gel doc system. In order to determine the sensitivity or the limit of detection (LOD) of MAP, serial dilutions of DNA from MAP 316 F were provided. The LOD of LAMP assay was 4 FG of DNA. The specificity of the assay was evaluated by testing three gram-positive bacteria including *Mycobacterium bovis AN5*, *Mycobacterium tuberculosis DT*, and *Mycobacterium avium subsp. avium*. Since LAMP correctly detected MAP, its specificity was 100%. The IS900 LAMP assay was derived from a study by Safi *et al.* (Safi *et al,*. 2014).(Table 1)

Table 1. Loop-mediated isothermal amplification (LAMP) primers used for detection of *Mycobacterium avium subsp. paratuberculosis* (MAP).

Target gene	Primer name	Primer sequence(5'-3')	Length (nucleotids)
IS900	B3	CACCTCCGTAACCGTCATTG	20
IS900	F3	GACGTCGGGTATGGCTTTCA	20
IS900	BIP	AGATGCGATTGGATCGCTGTGTTTTTTCCAGATCAACCCAGCAGAC	46
IS900	FIP	ATTAGCGGTCGAGTCGTCGCGTTTTGTGGTTGCTGTGTTGGATGG	45
IS900	LF	CGCCGGGCGGCCAATCTCC	19
IS900	LB	AAGGACACGTCGGCGTGGTC	20

Serum ELISA

Serum samples (n = 225) were tested using a commercial indirect ELISA kit (ParaCheck, Prionics AG, Zurich, Switzerland) based on detection of antibodies against protoplasmic MAP antigens, including a pre-absorption step with *Mycobacterium phlei*.

Statistical analysis

Laboratory data were analyzed using R software (http://www.r-project.org/). LAMP and ELISA were compared with culture in terms of sensitivity, specificity, and clinical accuracy.

Results

Culture

Despite the mentioned shortcomings of culture, fecal culture was selected as the gold standard in the diagnosis of MAP infection in this study. Samples with bacterial growth on HEYM with mycobactin J were regarded as positive. Samples with no bacterial growth after six months were considered negative. After about eight weeks, small colonies could be observed on the culture media. From the 225 collected fecal samples, six were found positive and 219 resulted negative. After DNA extraction, PCR was conducted and the presence of MAP in the colonies was confirmed. All colonies grown on HEYM with mycobactin J resulted positive in PCR.

Colony morphology

The colonies were initially small but grew to 3 mm in diameter over time. They appeared as white to pale yellow transparent, bright spheres with smooth to slightly rough surfaces (Fig. 1).

ELISA

From the 225 tested Serum samples, 19 were positive and 206 were negative. From the six fecal samples with positive culture, five had positive serum results. Moreover, 14 samples with negative culture results were found positive by ELISA.

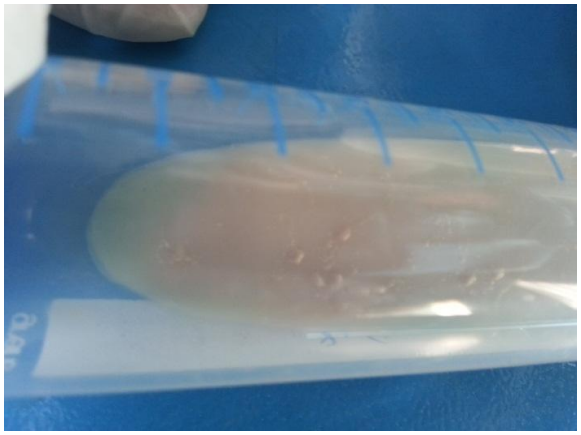

Fig. 1. Colonies of *Mycobacterium avium subsp. paratuberculosis* (MAP) on Herrold's egg yolk agar (HEYM) with mycobactin J.

LAMP

A total of 52 out of 225 collected samples turned out positive using LAMP. All samples with positive culture results were also positive which formed white precipitations, adding SYBR Green I. In addition, 46 samples with negative culture were found positive based on this method. Electrophoresis of LAMP amplified products demonstrated a typical ladder-like pattern and produced bands of different sizes (Fig. 2, 3). Furthermore, evaluation of the LAMP products with the naked eye under natural light revealed a white turbidity.

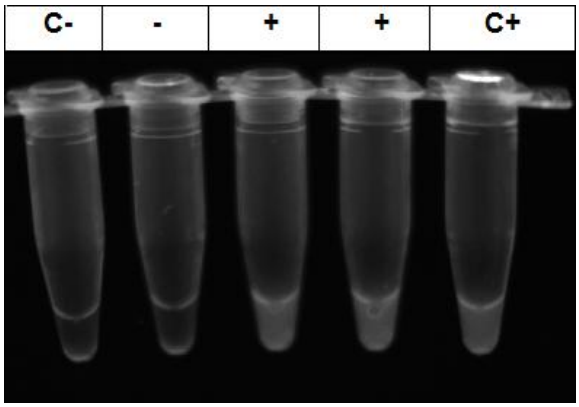

Fig. 2. Visual detection of MAP by loop-mediated isothermal amplification (LAMP) under UV light. C-: Negative control, -Negative sample, + Positive sample, C+: Positive control.

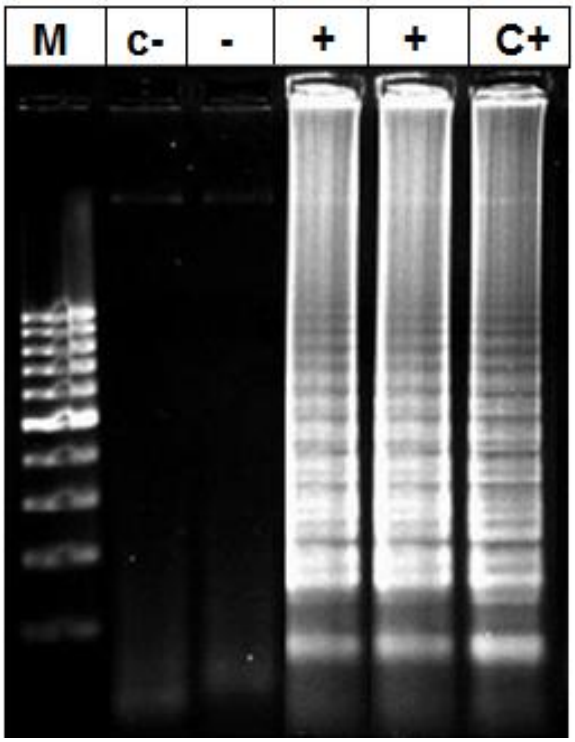

Fig.3. Electrophoretic detection of MAP by loop-mediated isothermal amplification (LAMP). M: Marker, C-: Negative control, -Negative sample, + Positive sample, C+:

Table 2. The efficiency of LAMP vs. culture on Herrold's egg yolk agar (HEYM).

lamp * Culture		
Diagnostic test	**Value**	**95% CI**
sensitivity	100.00 %	54.05 % to 100.00 %
specificity	79.00 %	73.00 % to 84.19 %
Positive Likelihood Ratio	4.76	3.68 to 6.16
Negative Likelihood Ratio	0.00	
Disease prevalence	2.67 %	0.99 % to 5.72 %
Positive Predictive Value	11.54 %	4.38 % to 23.45 %
Negative Predictive Value	100.00 %	97.87 % to 100.00 %

Table 2 and 3 compare LAMP and ELISA with culture in terms of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio of a positive test result (LR+), and likelihood ratio of a negative test result (LR-). Comparisons between LAMP and ELISA suggested the first to have higher sensitivity (100% vs. 83.33%) but lower specificity (79% vs. 93.61%). Table 4

compares the agreement between LAMP and ELISA with fecal culture. Table 5 compares the agreement between loop-mediated LAMP and ELISA.

Table 3. The efficiency of ELISA vs. culture on Herrold's egg yolk agar (HEYM).

ELISA * Culture		
Diagnostic test	**Value**	**95% CI**
sensitivity	83.33 %	36.10 % to 97.24 %
specificity	93.61 %	89.51 % to 96.46 %
Positive Likelihood Ratio	13.04	7.01 to 24.24
Negative Likelihood Ratio	0.18	0.03 to 1.07
Disease prevalence	2.67 %	0.99 % to 5.72 %
Positive Predictive Value	26.32 %	9.25 % to 51.20 %
Negative Predictive Value	99.51 %	97.31 % to 99.92 %

Table 4. The agreement between LAMP and ELISA with fecal culture.

Culture * ELISA					
Expected					
Agreement	**Agreement**	**Kappa**	**Std. Err.**	**Z**	**p value**
93.33%	89.34%	0.3747	0.0560	6.69	0.0000
Culture * lamp					
Expected					
Agreement	**Agreement**	**Kappa**	**Std. Err.**	**Z**	**p value**
79.56%	75.45%	0.1671	0.0369	4.53	0.0000

Table5. The agreement between LAMP and ELISA

Culture * ELISA					
Expected					
Agreement	**Agreement**	**Kappa**	**Std. Err.**	**Z**	**p value**
80.00%	72.35%	0.2767	0.0565	4.90	0.0000

Discussion

Much like other mycobacterial infections, effective control of paratuberculosis is difficult and interpretation of the test results is challenging due to lack of proper and rapid diagnostic tests, long latent period, undiagnosed subclinical cases, and lack of adequate knowledge about variety of MAP species (Whittington,. 2009; Soha *et al*,. 2007).

Currently, common methods for diagnosis of Johne's disease include: fecal culture, microscopic tests, ELISA, and a variety of PCR techniques. Fecal culture, with sensitivity of 30% to 50% and specificity of 100% is commonly used (Whitlock *et al*,. 2010), even though, due to the slow growth of MAP (8-16 weeks) and the potential risk of contamination of cultures, it can only be performed in reference laboratories. Use of serum ELISA in screening for Johne's disease in cattle is quite common. However, positive test results should be confirmed by PCR or culture. Different PCR methods are used for diagnosis of MAP, whose aim is amplification of specific bacterial sequences including: IS900, HspX, F57, and …. Despite high sensitivity and specificity, these methods require advanced and expensive equipments, and their correct interpretation relies on adequate skill and experience. As a result of these limitations, these methods are only used in fully equipped laboratories, with specialist staff, and cannot be performed in remote and deprived areas that require rapid identification of pathogens to prevent serious damage (Castellanos*et al*,.2012. Pinedo *et al*,.2008).

Therefore, there is a need for a simpler and more efficient method that can be performed in both fully equipped and ordinary and small laboratories, even by non-specialist staff.

In the present study, MAP was examined using optimized LAMP technique, in which IS900 gene was selected as the target sequence. Many studies have proposed IS900 as the most specific sequence for various PCR-based molecular tests. Thus, we used this sequence to design primers. The 6 primers designed were specifically amplified for reaction with 8 areas of target sequence in the online software for LAMP [Primer Explorer (htpp://primerexplorer.jp/elamp3.0.0/index.html)]. LAMP products were visually examined for opacity and assessed for changes by fluorescent SYBR Green I, 0.1% by UV light. In the present study, final duration of LAMP method was 1 hour that was much shorter compared to usual PCR methods. LAMP method applies an

isothermal temperature of 63 °C, provided by a heat block or thermo-mixer. Hence, unlike other usual PCR methods, there was no need for a thermo cycler, electrophoresis, gel-doc or ... LOD of the method was calculated as 10^{-8} or 4 femtogram and the analytical specificity as 100%. Following optimization of LAMP for evaluating the clinical efficacy of the test and comparing it with fecal culture as the gold standard and serum ELISA as the usual cattle screening method, 225 fecal samples and serum sample were taken from the herds of small, medium and large size commercial dairy farms. After bacterial DNA extraction from fecal samples and performing the diagnostic tests, most positive tests were diagnosed using LAMP and fecal culture had the fewest negative results. According to the statistical results, both LAMP and ELISA tests had a significant agreement with fecal culture. ELISA had a higher ($P<0.001$, $k=0.374$) and LAMP had a lower agreement ($P<0.001$, $k=0.167$). Furthermore, the agreement among the three tests was assessed. Accordingly, ELISA and LAMP tests had 80% agreement with each other ($P<0.001$, $k=0.276$). Meanwhile, PCR showed a 79.5% agreement with culture. Of the 52 positive LAMP samples, only 6 were reported positive in culture. Also, all positive culture samples were positive with LAMP method. Thus, the number of positive cultures with negative LAMP result was found zero, which shows a 100% clinical sensitivity of LAMP compared to the gold standard. Low positive results in fecal culture may be attributed to intermittent bacterial shedding, low detection sensitivity in conventional culture method used, low number of bacteria in defecation or low number of cattle in clinical and advanced stages of the disease. As noted in several studies, one of the main limitations in fecal culture is its low clinical sensitivity, which varies in these studies (Möbius *et al*,.2008). Generally, recent studies suggest higher sensitivity for various IS900-PCR-based tests. Ineffectiveness of PCR inhibiting factors in feces (which is a well-known problem in PCR) on LAMP method can also be considered another factor in interpreting a greater number of positive results and an explanation for none of the positive culture samples being negative (Khare *et al*,.2004; Melville *et al*,. 2014). These results can be confirmed by taking fecal samples with 3 months from the first round (Tiwari *et al*,.2006). Furthermore, ELISA showed an acceptable agreement with the molecular technique mentioned, although its level was not high. The finding of low agreement between PCR-based techniques with ELISA method has also been reported in previous studies (Pinedo *et al*,.2008).

In a study comparing fecal culture with serum ELISA and direct PCR fecal samples, 250 serum and fecal samples from dairy cattle with unknown status of disease were examined. Of the 250 samples, 67 in fecal culture, 74 in PCR, and only 25 in ELISA were reported positive. In this study, given that fecal culture was considered the gold standard, clinical sensitivity of PCR was reported higher compared to ELISA, so that clinical sensitivity and clinical specificity of PCR were 70.2% and 85.3% respectively, compared to 31.3% and 98% in ELISA. In the mentioned study, higher speed and sensitivity of PCR compared to culture and ELISA methods are emphasized, and PCR is proposed as an appropriate alternative to these two screening methods for Johne's disease in dairy cattle (3). Another study investigated the relationship between results of serum ELISA, fecal culture, and Nested PCR methods to identify LAMP in milk and fecal samples. They examined samples of milk, blood and fecal from 328 cattle, in 4 herds with known disease conditions. Sixty-one cattle (%18.6) were reported positive in parallel interpretation of tests. In a paired comparison, agreement between test results was reported poor; and good in other states. Fecal culture and Nested-PCR showed the highest agreement, and the least agreement was found between PCR and ELISA. These results concur with the present study results (Möbius *et al*,. 2008). In a study, sensitivity of ELISA was estimated between 9% and 17%, in animals with subclinical infection and low MAP

defecation (pinedo *et al*,. 2008). In another study, this was reported between 15% and 88%, depending on progress of disease toward clinical stages. In late stages of the disease, sensitivity of serological tests may be very low (between 10% and 25%) due to immune anergy (pinedo *et al*,. 2008).In the present study, like other similar studies, the main problem and limitation was absence of a perfect gold standard, which determines the actual status and correct stage of infection in animals. Consequently, the number of positive results cannot be definitely considered false, and in this situation, interpretation is challenging. In fact, low agreement and mismatch between results in most tests provides necessary information for using a parallel combination of several tests in assessing different stages of the disease. According to results obtained in this and other studies, combination of ELISA and PCR-based tests can increase overall sensitivity in detecting paratuberculosis infection. Given advantages of LAMP, including: high sensitivity and specificity, low cost, lack of need for advanced equipments, visual assessment, and positive results, compared to other available PCRs; it is recommended that LAMP be used for direct identification of contaminated livestock through fecal samples. Furthermore, due to inability of ELISA and other serological tests in detecting early stages of the disease, and time-consumption and high cost of culture, LAMP should be used alongside ELISA in cattle-wide screening for disease.

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