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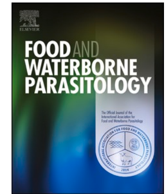

Design and evaluation of loop-mediated isothermal amplification for rapid detection of *Enterocytozoon bieneusi*

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A R T I C L E I N F O

Keywords:
Enterocytozoon bieneusi
Loop-mediated isothermal amplification
Point of care
Real-time PCR

A B S T R A C T

Enterocytozoon bieneusi is one of the most prevalent microsporidia species, responsible for more than 90% of human and animal microsporidiosis. Microsporidia species, particularly *E. bieneusi,* are frequently reported from waterborne and foodborne outbreaks. Therefore, early detection is crucial in clinics and outbreak investigations. This study aimed to design a loop-mediated isothermal amplification (LAMP) for rapid detection of *E. bieneusi.* Total DNA was extracted from 30 *E. bieneusi* –positive samples, which had been confirmed with nested PCR. LAMP primers were designed based on the identical fragment of small subunit ribosomal RNA (*SSU rRNA*) gene. LAMP reactions were performed at 63 °C for 60 min. The sensitivity and specificity of the assay were analyzed and the results of amplification were compared to real-time PCR. Our results showed that the LAMP assay successfully amplified 25/30 (83.3%) samples. The specificity results indicated no false positive with other microorganisms. Furthermore, the LAMP method exhibited a sensitivity (limit of detection, LoD) as low as 34 ag/μL of total DNA. Compared to the LAMP assay, real-time PCR was able to detect all 30 nested PCR-positive samples. Our findings showed that the LAMP assay was able to detect 83.3% of *E. bieneusi*-positive samples. Although the current assay was not able to detect all nested PCR-positive samples, the lack of need for specific instruments, rapid processes, and high specificity makes LAMP assay a suitable tool for screening.

1. Introduction

Microsporidia are a group of obligate intracellular eukaryotes with more than 1400 species, of which 17 species are responsible for human microsporidiosis (Li et al., 2019; Ruan et al., 2021). More than 90% of human and animal microsporidiosis is caused by *Enterocytozoon bieneusi* (Deng et al., 2018). Contamination of water and food resources with *E. bieneusi* spores is the main sources of human infections (Javanmard et al., 2018; Li et al., 2019).

The infection in immunocompetent individuals is asymptomatic and self-limited, but in immunocompromised patients, such as

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https://doi.org/10.1016/j.fawpar.2024.e00225
Received 1 November 2023; Received in revised form 3 March 2024; Accepted 11 March 2024
Available online 13 March 2024
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Table 1
Sequences of LAMP primer sets designed for *E. bieneusi* detection in the present study.

Parasite	Target gene	Primers 5′-3′
E. bieneusi	*SSU rRNA*	F3: AGCCGCGGTAACTCCAACT B3: TGCAGGAACTACAGCGGTG FIP: GCATTCACGACTACGGACCCGAGTGTCTATGGTGGATGCTGC BIP: AGGCGACACTCTTAGACGTATCCCTGCCTTCGTCCTTGATCCT

cancer patients, HIV/AIDS individuals, and transplant recipients, gastrointestinal tract disorders and prolonged diarrhea, as well as disseminated infections are common (Fayer and Santin-Duran, 2014; Ghoyounchi et al., 2017; Li et al., 2019; Karimi et al., 2020; Ruan et al., 2021). Regarding the importance of opportunistic infections, early detection is necessary for treatment strategy plans in clinical settings. In addition, concerning the importance of waterborne and foodborne outbreaks due to *E. bieneusi,* rapid detection at the beginning of the outbreaks is crucial (Abhari et al., 2023; Nemati et al., 2023).

There are couple of diagnostic techniques for parasitic diseases with different efficiency. The first line detection method for intestinal parasites is coproscopy. However, due to the high error possibility, time-consuming, and the need for skilled microscopist, new diagnostic techniques have been developed (Abhari et al., 2023; Abu-Madi et al., 2017; Garedaghi, 2020).

Loop mediated isothermal amplification (LAMP) is one of the nucleic acid amplifications (NAA) techniques, which was firstly developed by Notomi et al. and patented by Eiken Chemical Co., Ltd., that has been popular due to its simplicity and rapid process (Abhari et al., 2023; Notomi et al., 2000). Unlike PCR, LAMP does not require thermal cycles and can be performed at a constant temperature in a single step (Avendaño and Patarroyo, 2020). Therefore, because of the simplicity, rapidity, no need for expensive equipment, and high sensitivity, LAMP has the potential to be a point of care (PoC) test, particularly for screening approaches (García-Bernalt Diego et al., 2021). Current study aimed to design and fabricate a LAMP assay for rapid detection of *E. bieneusi* among clinical and environmental samples, and to compare the results with real-time PCR.

2. Materials and methods

2.1. Ethical approval

All experimental protocols were in accordance to the ethical principles and the national norms and standards for conducting Medical Research in Iran. This study was approved by the ethical standards (IR.SBMU.MSP.REC.1400.571 and IR.SBMU.RIGLD.REC.1401.027) released by the Ethical Review Committees of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

2.2. Sample sources and DNA extraction

In this study, a total of 30 samples, which had been identified positive for *E. bieneusi* using nested-PCR (Mohammad Rahimi et al., 2021), were retrieved from the stock bank of the Parasitology and Water Research laboratory of the Foodborne and Waterborne Diseases Research Center at Shahid Beheshti University of Medical Sciences, Tehran, Iran. The samples included 19 animal stool samples (cattle, chicken, horses, sheep, and goat), 9 vegetables samples (parsley, garden cress, mint, basil, chives) and 2 wastewater samples (oil wastewater). Briefly, 250 μL of samples, which were suspended in sterile PBS (pH = 7.5) was transferred to 1.5-mL tubes. After centrifugation at 2500 ×*g* for 5 min, supernatant was discarded, and DNA was extracted from the remained pellet using a commercial spin column-based DNA extraction kit (Yekta Tajhiz Azma, Tehran, Iran). Finally, extracted DNA was stored at - 20 °C until use in real-time and LAMP PCR.

2.3. Primer design for LAMP

To design LAMP PCR primers for *E. bieneusi*, small subunit ribosomal RNA (*SSU rRNA*) gene was selected as an appropriate target. The LAMP primers were manually designed based on the *SSU rRNA* gene using BioEdit and GeneRunner software. The accession numbers of reference sequences for primer designing were: OM305003.1, OM033625.1, and OM033624.1. To confirm the non-specific amplification of other intestinal microsporidia, designed primers were checked using primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) for *Encephalitozoon intestinalis, E. hellem*, and *E. cuniculi*. As a result, four LAMP primers, targeting six regions of the target gene, were designed including Eb-F3, Eb-B3, Eb-FIP, and Eb-BIP (Table 1).

2.4. LAMP assay

To confirm the specific amplification of primers, PCR assay using two outer primers including Eb-B3 and Eb-F3, which amplifies ~274-bp fragment of the target gene, was employed. The PCR amplification was performed in a 15 μL volume consisted of 7.5 μL of 2× Taq red master mix (Ampliqon, Odense, Denmark), 10 ρM of each forward (Eb-F3) and reverse (Eb-B3) primers, and 2 μL of DNA templet. The cycling conditions were: an initial denaturation step of 94 °C for 4 min, followed by 40 cycles of 94 °C for 1 min, 60 °C for 45 s, and 72 °C for 1 min. The final extension cycle was 72 °C for 5 min.

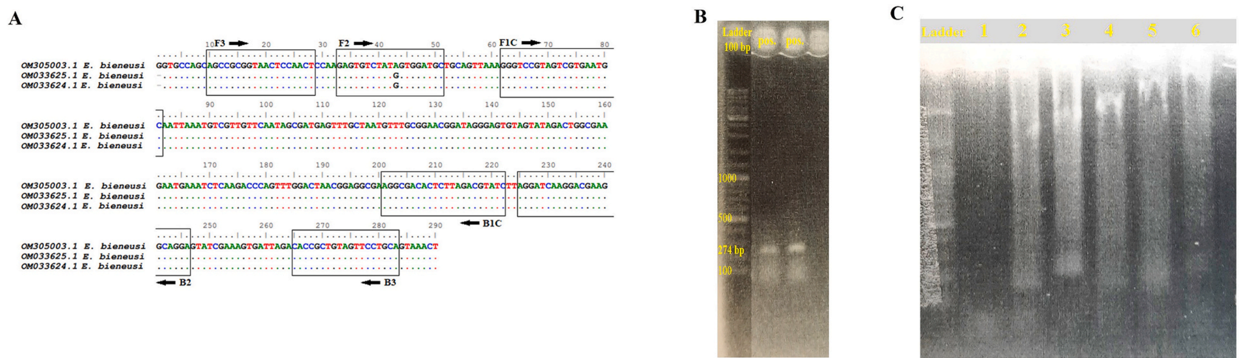

Fig. 1. A) Schematic representation of the LAMP primer design. B) The PCR result of *E. bieneusi* with Eb-F3 and Eb-B3 primers (the figure was cropped from an original figure as suppl Fig. 1). C) Different temperatures, which were employed to optimize the LAMP assay. 1) 65 °C; 2) 64 °C; 3) 63 °C; 4) 62 °C; 5) 61 °C; 6) 60 °C.

The LAMP reaction was performed in a Rotor-Gene Q (QIAGEN, Germany) real-time instrument by Eva Green fluorescence dye for *E. bieneusi*-positive samples. The amplification was carried out in a 25 μL PCR reaction mix containing: 40 pmol of each Eb-FIP and Eb-BIP primer, 5 pmol of each Eb-F3 and Eb-B3 primer, 8 U of *Bst* 2 DNA polymerase (New England Biolabs, USA), 1.4 mmol/L of deoxynucleoside triphosphates (dNTP), 2.5 μL 10× isothermal amplification buffer (New England Biolabs, USA), 6 mmol/L of $MgSO_4$, and 2 μL of extracted DNA. Distilled water and a previously confirmed DNA for *E. bieneusi* were employed as negative and positive controls, respectively. To identify the optimal temperature and time for LAMP amplification, the reactions were conducted in 60–65 °C. The optimal time was evaluated every 15 min from 30 to 120 min. Specific melting curve indicated specific target amplification. The LAMP products were electrophoresed on a 1.2% agarose gel and visualized using ethidium bromide to confirm LAMP reaction.

2.5. Specificity of LAMP primers

To analyze the specificity of the designed primers, we conducted LAMP assay on the DNA of a panel of protozoa, helminths, fungi, and bacteria, which were available in our biobank. These microorganisms include *Giardia lamblia*, *Cryptosporidium parvum*, *Toxoplasma gondii*, *Blastocystis*, *Acanthamoeba*, *Trichinella britovi, Fasciola* spp., *Dicrocoelium dendriticum, Trichostrongylus, Clostridium difficile, C. perfringens, Escherichia coli, Staphylococcus aureus, Shigella* spp., *Klebsiella* spp., *Pseudomonas aeruginosa, Candida albicans, C. glabrata, Saccharomyces cerevisiae,* and *Geotrichum candidum.* Furthermore, to cover all other genera of microorganisms that may be found in stool samples, LAMP assay was also performed on ten stool samples from apparently healthy persons, which were negative for *E. bieneusi* using nested-PCR.

2.6. Sensitivity of LAMP PCR

To evaluate sensitivity of the LAMP assay, initial DNA concentration of a *E. bieneusi*-positive stool sample was measured with NanoDrop (Multiskan SkyHigh Microplate Spectrophotometer; Thermo Scientific™, USA). After that, 10 serial dilutions from $\log 10^{-1}$ to $\log 10^{-10}$ were prepared from a *E. bieneusi* positive DNA (34 ng/μL) and run together with original concentration and negative control. Finally, LAMP PCR was performed for each of diluted DNA sample and the sensitivity was reported as the limit of detection (LoD) for total DNA.

2.7. Real-time PCR

To compare the results of LAMP assay, real-time PCR was carried out using specific primers targeting internal transcribed spacer (*ITS*) gene (Verweij et al., 2007). Real-time PCR was performed using Rotor-Gene Q real-time instrument in a 20 μL total volume containing 10 μL of 2× SYBR Green real-time PCR master mix (Ampliqon, Denmark), 10 ρM of each primer, and 2 μL of DNA. Thermal profile for amplification consisted of 95 °C for 10 min followed by 50 cycles: 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, and ramping from 70 °C to 95 °C at $1°Cs^{-1}$.

3. Results

3.1. LAMP assay

The result of in silico analyses and conventional PCR with Eb-F3 and Eb-B3 primers showed a ~ 274-bp band in electrophoresis, which verifies the specific binding of primers to the target gene. To determine the results of LAMP assay, amplification plots generated by the real-time PCR instrument were considered. The results showed that the 63 °C for 60 min is the best condition for LAMP assay

Table 2
LAMP results according to sample sources.

Sources	Real-time PCR-positive	LAMP-positive
Animals	19	16
Wastewaters	2	2
Vegetables	9	7
Total	30	25

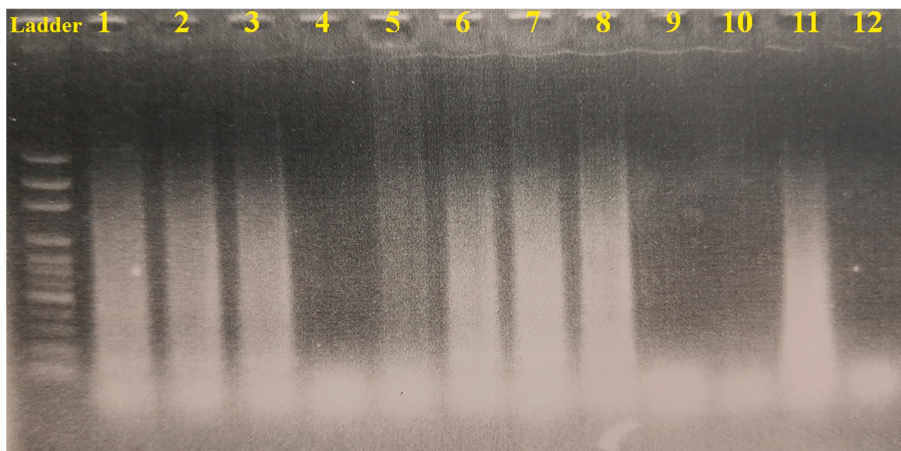

Fig. 2. Electrophoresis of LAMP products amplified from *E. bieneusi* DNA. 1–10) DNA samples; 11) positive control; 12) negative control (distilled water).

(Fig. 1A, B, and C, Suppl Fig. 1). The validity of amplification was evaluated using melting curve analysis. The average time and melting curve values were 32.3 ± 8.8 min (17.5–46) and 89.5 °C ± 0.8 (88.5–91.2), respectively. Accordingly, from 30 nested-PCR positive samples, 25 (83.3%) were positive by the LAMP method (Table 2). All LAMP products were electrophoresed on agarose gel that the results confirmed the results of LAMP assay (Fig. 2).

3.2. Specificity of LAMP primers

There was no evidence for any amplification by the LAMP assay, when the primers were evaluated with DNA of protozoa, helminths, fungi, and bacteria, as well as total DNA extracted from 10 apparently healthy samples (Fig. 3A).

3.3. Sensitivity of LAMP PCR

The sensitivity analysis of the LAMP assay showed that the test was positive for *E. bieneusi* until the log 10^{-9}. Accordingly, the test was positive in nine concentrations from 3.4 ng/μL to 34 ag/μL of total DNA, which was extracted from stool sample infected by of *E. bieneusi* (Fig. 3B).

3.4. Real-time PCR

Real-time analysis showed that All 30 *E. bieneusi*-positive samples were confirmed with real-time PCR.

4. Discussion

Microsporidia represent a class of intracellular pathogens with approximately 1400 species, of which 17 species are supposed to be human pathogens (Li et al., 2019; Ruan et al., 2021). Microsporidiosis affects individuals with intact immune systems, children, travelers, and immunocompromised individuals such as HIV/AIDS patients, transplant recipients, etc. (Fayer and Santin-Duran, 2014; Ghoyounchi et al., 2017; Li et al., 2019; Ruan et al., 2021). *E. bieneusi* is transmitted to humans through various routes including fecal-contaminated food, water, and environment samples. Regarding the numerous clinical reports and outbreaks, microsporidiosis is now considered as a public health issue (Javanmard et al., 2018; Li et al., 2019). For example, an outbreak of *E. bieneusi* occurred in Sweden in 2009, which was resulted from contaminated cucumber (Decraene et al., 2012). Furthermore, a recent outbreak of *E. bieneusi* diarrhea was occurred in a workplace canteen in Denmark, which was linked to the consumption of open sandwich lunchboxes (Michlmayr et al., 2020). These instances along with the frequent reports of *E. bieneusi* infections in both humans (Ahmadi et al., 2023;

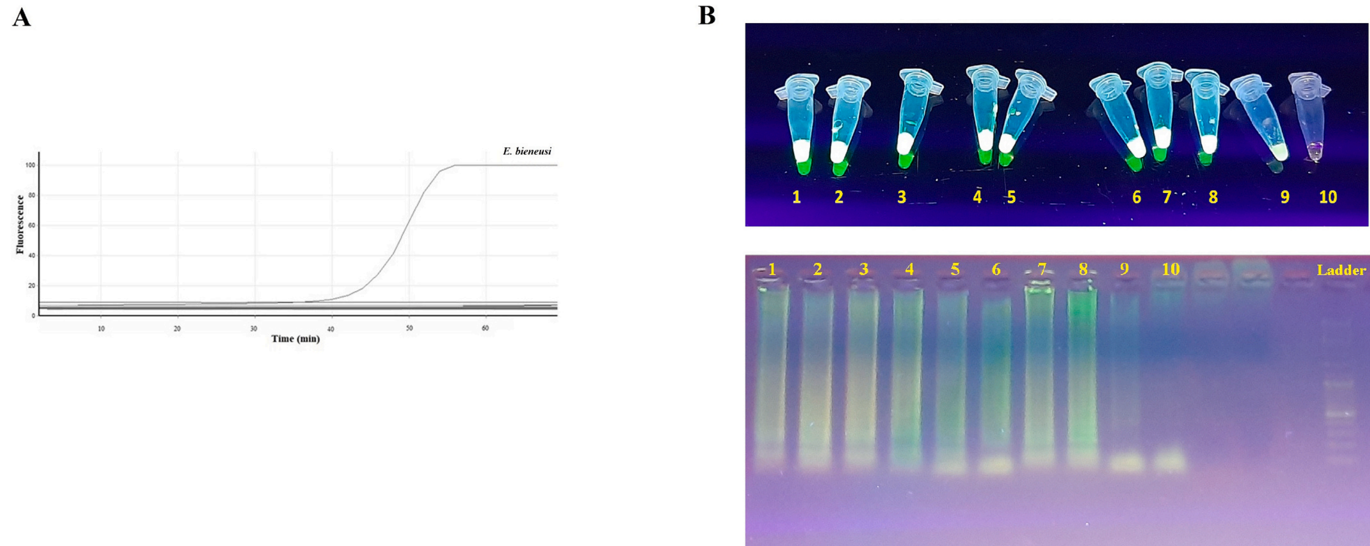

Fig. 3. A) The analytical specificity of LAMP primers evaluated using non-*E. bieneusi* DNA. B) The UV imaging and gel electrophoresis of analytical sensitivity of each LAMP reaction evaluated using 10 concentrations of genomic DNA ranging from 3.4 ng/μL to 3.4 ag/μL. 1) 3.4 ng/μL; 2) 0.34 ng/μL; 3) 34 pg/μL; 4) 3.4 pg/μL; 5) 0.34 pg/μL; 6) 34 fg/μL; 7) 3.4 fg/μL; 8) 0.34 fg/μL; 9) 34 ag/μL; 10) 3.4 ag/μL.

Shen et al., 2020) and livestock (Bilgin et al., 2020; Chang et al., 2020; Mohammad Rahimi et al., 2021; Zhang et al., 2020) highlight the zoonotic concern due to microsporidia infections and the necessity for rapid diagnostic tests.

Recently, PoC methods have attracted considerable interests, and many efforts have been directed toward developing PoC tests, particularly in the field conditions. One of highly appealing PoC tests is LAMP method. The isothermal nature of LAMP eliminates the need for a thermocycler or sophisticated equipment (Avendaño and Patarroyo, 2020; García-Bernalt Diego et al., 2021). Moreover, this method detects targeted agent in just a half an hour to an hour with high sensitivity and accuracy (Avendaño and Patarroyo, 2020; García-Bernalt Diego et al., 2021). Therefore, LAMP assay seems to be a good alternative tool for screening strategies, especially in microbial outbreaks.

LAMP assay for detection of *E. bieneusi* is very rare in literature. In 2015, a study was conducted to design LAMP primers for *E. bieneusi*, and the results was compared to PCR methods (Nur Su'aidah Nasarudin et al., 2015). The results revealed that the LAMP method exhibited a sensitivity of 94% and a specificity of 88% compared to the PCR method (Nur Su'aidah Nasarudin et al., 2015). In contrast, our current study, the LAMP technique was compared with the real-time method and the results showed that the LAMP method had a sensitivity of 83.3%, whereas it achieved a specificity of 100% in accurately diagnosing *E. bieneusi*. Although the diagnostic sensitivity of LAMP seems to be lower in comparison to real-time, its diagnostic capabilities, along with the fact that it can be performed using a simple water bath, which are readily available even in resource-limited areas, make it a suitable tool for screening of infectious diseases. In addition, it should be considered that the real-time PCR targets an identical fragment of the ITS gene (Verweij et al., 2007), while due to limitation in designing primers for such a short gene like ITS in *E. bieneusi,* LAMP primers in our study were designed for a conserved fragment of the *SSU rRNA* gene. However, to enhance the sensitivity of LAMP assay, it is recommended to investigate other conserved multi-copy genes as potential candidates for primer designing.

5. Conclusions

In the current study a LAMP assay was successfully fabricated for rapid detection of *E. bieneusi.* The current test is able to detect *E. bieneusi* in 34 ag/μL of total DNA extracted from a stool sample. Our findings suggest that although the LAMP assay was not able to detect all nested PCR-positive samples, the lack of need for specific instruments, rapid processes, and high specificity makes LAMP assay a suitable tool for screening *E. bieneusi* in stool samples compared to nested-PCR and real-time PCR.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2024.e00225.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors thank all members of the Foodborne and Waterborne Diseases Research Center for their supports. This study was financially supported by the Shahid Beheshti University of Medical Sciences, Tehran, Iran (grant no: 43002426).

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