SHORT COMMUNICATION

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An accurate, rapid and simple loop-mediated isothermal amplification method for *Explanatum explanatum* detection in animals

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Abstract

Paramphistomosis is an infectious disease caused by the liver flukes and it is associated with heavy loss of ruminant's population. *Explanatum explanatum* is a digenetic trematode commonly affecting domesticated ruminants. The available methods for pathogen detection are laborious and expensive and offer limited specificity; thus, considered not suitable for post mortem pathogen detection, surveillance and prevalence studies. New detection techniques offering simplicity, specificity and rapidity are absolutely needed. We have designed a loop-mediated isothermal amplification (LAMP) based polymerase chain reaction method, targeting a sequence of the *explanatum* species, using a primer pair from the internal transcribed spacer-2 region. The DNA from adult flukes belonging to *explanatum* species was isolated from infected livers and used to optimize the LAMP assay. The specificity and sensitivity of the LAMP assay were evaluated and found highly efficient in species-specific DNA detection with the sensitivity to detect 50.00 pg DNA in a 25.00 µL reaction mix. The procedure has the potential to be adapted for stool samples for field detection and disease surveillance/prevalence in rural and unprivileged areas.

Introduction

Pakistan being an agricultural land largely depends on livestock income. Livestock and its by-products like milk, meat, dung, etc., contribute significantly in the national economy and a large portion of farmers depend on income, which they earn through livestock farming. Buffalo farming is a popular business in Asia and especially in Pakistan due to the high yield of milk. The share of buffalo milk is only 12.00% of the total milk produced around the world; but, it is around 38.00% of the milk produced in Asia and 66.00% of the milk produced in Pakistan. Domesticated buffalo (*Bubalus bubalis*) is the main contributor to milk. There are approximately 23.50 million water buffaloes in Pakistan.[1-3] Water buffaloes are generally kept in water and mud-rich surroundings, where they can wallow. This environment equally suits to water and mud snails, which serve as an intermediate host for a variety of trematode parasites.[4] The livestock industry is largely affected by a number of helminthic parasite infections, resulting in a heavy loss in livestock production.

Paramphistomosis which is associated with a significant loss in ruminant's population is an infectious disease of trematodes.[5-8] Among them the *Explanatum explanatum* is a common digenetic trematode, largely affecting domesticated ruminants. The *E. explanatum* infection is associated with heavy losses of livestock because it produces a large number of eggs and can survive in all seasons.[9] Bile duct of ruminants is generally colonized by adult stages of *Explanatum* spp. where they feed the blood; while, immature stages of *Explanatum* spp. feed blood in the small intestine.[10-13] Existing estimates of livestock losses made by liver flukes are mainly obtained by post mortem pathogen detection using microscopic techniques. However, these data are limited due to the complex procedures, requiring sophisticated instruments and laboratory infra-structure. A rapid, reliable, low-cost, specific and field-friendly method for the detection of *E. explanatum* is an absolute requirement for the post mortem pathogen surveillance, prevalence and characterization studies of the liver flukes infections.

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We have optimized a highly efficient, accurate and reliable loop-mediated isothermal amplification (LAMP) based polymerase chain reaction (PCR) method for the detection of *E. explanatum*. The LAMP assay was first introduced in the year 2000,[14] and gained immense attention and appreciation due to its simplicity. This method offers significant advantages over conventional PCR and/or serology-based methods due to its simplicity, ease and rapidness. The test can be carried out within 60 min and it does not require sophisticated instruments; thus, it can be performed in low resource field settings. We have demonstrated an optimized method for a rapid and reliable detection of liver flukes, *i.e.*, *E. explanatum* which is absolutely adaptable for field-based post mortem pathogen surveillance and prevalence studies.

Materials and Methods

Collection of *E. explanatum* flukes. The flukes were collected from infected livers of three buffaloes from a slaughterhouse in the city of Lahore, Pakistan, and transported to the laboratory on ice. The livers were dissected to recover flukes in the biliary ducts. Multiple adult flukes were collected from each liver and examined for their morphology, size and weight. The genus and species of the flukes were confirmed by PCR method using internal transcribed spacer-2 (ITS2)-ribosomal DNA sequences as previously described.[10]

Genomic DNA isolation. Recovered flukes from livers were washed repeatedly with phosphate buffered saline (PBS) and preserved in 70.00% ethanol at – 80.00 ˚C. For DNA extraction from flukes, 2.00 mg of tissue from each fluke was weighted and rinsed thrice with distilled water for 5 min. Each tissue section was then lysed using 50.00 µL of lysis buffer (50.00 mM KCL, 10.00 mM Tris (pH= 8.30), 2.50 mM $MgCl_2$, 0.045% Nonidet p-40 and 0.45% Tween-20) containing 10.00 mg mL^{-1} proteinase K (New England BioLabs, Ipswich, USA) for 98 min at 60.00 ˚C, followed by inactivation of proteinase K for 15 min at 94.00 ˚C and then stored at – 80.00 ˚C until used.

Design of LAMP primers. The oligonucleotide LAMP primers were designed targeting the sequences of DNA fragments of the nuclear ITS2 region including partial 5.8S and 28S ribosomal RNA genes (AB743577.1), as previously found to be highly conserved in *E. explanatum*.[11,15] Several primer sets were suggested via online LAMP primer design software PrimerExplorer (version 5.0; Eiken Chemical, Tokyo, Japan) and suggested primer sets were analyzed manually based on the criteria described in A Guide to LAMP primer designing (https://primerexplorer.jp/e/v4_manual/pdf/PrimerExplorerV4_Manual_1.pdf. Selected primer sequences are given in Table 1.

Loop-mediated isothermal amplification-based PCR reactions. Ideal conditions for LAMP-based PCR were adjusted, followed by testing a variety of combinations for a PCR reaction. The best amplification conditions based on the most vivid color change, intensity of the band on agarose gel and reproducibility of results were found with the reaction mixture containing 0.20 M of outer primers (F3 and B3), 1.60 M inner primers (FIP and BIP), 8.00 U of *Bst* 2.0 warmStart® DNA polymerase (New England Biolabs), 1X isothermal amplification buffer (New England Biolabs), 8.00 mM $MgSO_4$ (New England Biolabs), 0.80 M betaine (Sigma-Aldrich, St. Louis, USA), 120 µM hydroxy naphthol blue (Sigma-Aldrich), 1.40 mM each dNTP (New England Biolabs) and 1.00 µL of template DNA or sterile distilled water (as a negative control), in final reaction volume of 25.00 µL at the temperature of 62.00 ˚C. Reaction was terminated by heating at 80.00 ˚C for 5 min. Parameters were individually examined for their effectiveness in three independent attempts.

Detection limit and specificity of the LAMP assay. Ten-fold serial dilutions of template DNA ranging from 50.00 ng μL^{-1} to 5.00 pg μL^{-1} were made in order to determine the detection limit of the LAMP assay. Also, to evaluate the specificity of the test, irrelevant DNA mix (DNA from *Toxoplasma gondii*, *Leishmania donovani* and *Escherichia coli*), 50.00 ng from each organism was used as a template DNA. The products of LAMP-based PCR were examined visually for color change violet (-ve) to sky-blue (+ve) and analyzed by 1.50% agarose gel electrophoresis with 0.01% ethidium bromide (Sigma-Aldrich).

Table 1. Nucleotide sequences of loop-mediated isothermal amplification primers for 18S ribosomal RNA.

Primer	Primer sequence (5′ - 3′)	Length (bp)
F3	TGATTTCCTCTGTGGTTCGC	20
B3	CGGACAGCAATAGCATCTCA	20
FIP	TCTGCGCTACCAGCAAGACG-GTGCCAGATCTATGGCGTT	39
BIP	GCGGTAGAGTCGTGGCTCAAT-ACACTGACAAAGGCACAACA	41

F3 and B3: Outer primers; FIP and BIP: Inner primers; LF and LB: Loop primers; The FIP primer was consisted of F2 and the complementary strand of F1 (F1c); The BIP primer was consisted of B2 and the complementary strand of B1 (B1c).

Results

Livers of buffalo were collected from a local slaughterhouse and examined for the flukes infection; bile duct was ruptured to reveal flukes and morphology of the collected flukes was also studied (Fig. 1).

The efficiency of the LAMP-based PCR reaction was demonstrated in Figure 2, where eight samples containing different template DNA, acquired from eight flukes of *E. explanatum*, were shown to be efficiently detectible. Sensitivity and specificity of the LAMP-based PCR assay were also evaluated using irrelevant template DNA and serially diluted template DNA, respectively. The fluke's template DNA was serially diluted ten-fold yielding 50.00 ng, 5.00 ng, 500 pg, 50.00 pg and 5.00 pg DNA and used as

a template DNA in the separate PCR vials to assess the detection limit of the LAMP assay. Whereas, irrelevant DNA mix acquired from *Toxoplasma gondii*, *Leishmania donovani* and *Escherichia coli* was used as a template DNA to test the specificity of the LAMP assay (Fig. 2B).

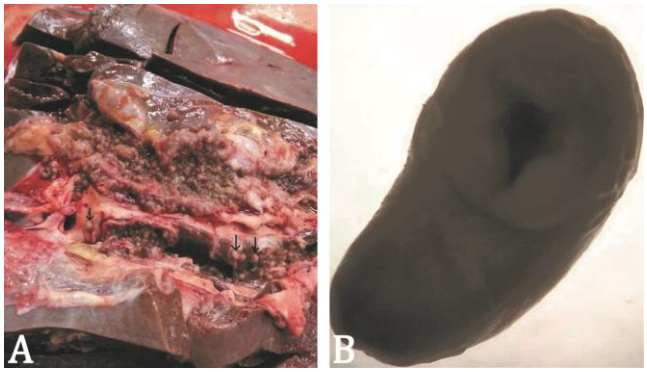

Fig. 1. A) The presentation of an infected liver with *E. explanatum* flukes. An infected ruminant's liver; where, the bile duct is ruptured to reveal colonized flukes. There are a number of flukes visible in the picture; whereas, few flukes are shown by the arrows. **B)** Morphology of a single fluke.

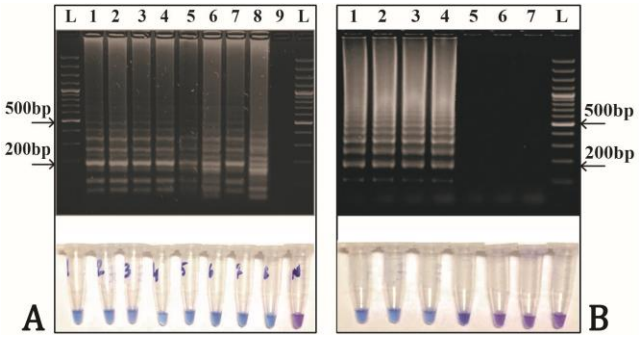

Fig. 2. Detection of primer specific loop-mediated isothermal amplified (LAMP) products. Upper panel shows detection of the product on agarose gel; while, the lower panel shows visible color changes of PCR reaction mix. **A)** Detection of eight samples (lanes: 1-8) containing template DNA from *E. explanatum*, ultra-pure water (lane: 9) was used as a negative control. The samples were run in parallel with the 100 bp DNA ladder. The primer-specific product can be clearly seen at the position of 200 bp (product size: 196 bp) in lanes 1-8; while, negative control (lane 9) shows no DNA. Similar results were shown in the lower panel, where hydroxy naphthol mediated visible color change (violet to sky-blue) indicating eight samples (1-8) as PCR positives (sky-blue) and negative control as a PCR negative (violet). **B)** Upper panel shows detection of primer specific LAMP product on agarose gel, where template DNA was either from *E. explanatum* (lanes: 1-5) or a DNA mix from *Toxoplasma gondii*, *Leishmania donovani* and *Escherichia coli* (lane 6) and ultra-pure water was used as a negative control (lane 7). Samples containing serially diluted template DNA (50.00 ng, 5.00 ng, 500 pg, 50.00 pg and 5.00 pg) were found detectable up to 50.00 pg DNA (lanes 1-4); while, 5.00 pg DNA (lane 5) found undetectable. Samples containing irrelevant DNA mix and water were also found undetectable. Same detection pattern was also observed by color changes of PCR reaction mix (lower panel).

We found that the LAMP assay can efficiently detect up to 50.00 pg DNA in a 25.00 µL volume and it did not show any unspecific amplification (Fig. 2B).

In this study, we demonstrated an accurate, rapid and simple detection of ruminant liver parasites using a LAMP-based PCR method. We have utilized DNA sequences of the ITS2 region including partial 5.8S and 28S ribosomal RNA genes (AB743577.1). The ITS2 sequence has been shown to serve as a marker for species identification of amphistomes[11] and it was already successfully used for the detection of *E. explanatum*.[10-12,15] To keep the assay simple and low cost, we have only used four primers including F3, B3, FIP and BIP consisting of 120 base pairs in total. For simplicity of detection, visual detection of PCR positive and negative vials was carried out using hydroxy naphthol blue.[16]

Discussion

The recent development made in the LAMP-based PCR assays for the detection of helminths including trematodes of different families has a significant importance for their applications in laboratory diagnosis, field surveys and surveillance of parasites.[17] The LAMP-based PCR executes better results than PCR and/or parasitological methods.[18-20] We have tried to build a rationale of LAMP-based PCR for the detection of *E. explanatum* for its applications in low-resource field based surveillance studies. Our results showed that the LAMP-based PCR assay can accurately and specifically detect the DNA extracted from flukes. All the samples were efficiently detected by LAMP-based PCR. The positive samples confirmed by agarose gel electrophoresis were found corresponding to visual detection using hydroxy naphthol blue, where sky-blue (+ve) and violet (-ve) can be easily distinguished by the naked eye (Fig. 2A). Specificity of the LAMP assay was also assessed using irrelevant DNA samples, a DNA mix of *Toxoplasma gondii*, *Leishmania donovani* and *Escherichia coli* was used as irrelevant DNA samples, and we found that LAMP-based PCR has not amplified any unspecific DNA; thus, it was not detectable by agarose gel electrophoresis and corresponding vials were also looked negative (violet) by visual observation (Fig. 2B). Serially diluted DNA samples were also run in parallel for determination of detection limit of the LAMP assay. The DNA up to the dilution of 50.00 ng μL^{-1} was found detectable (Fig. 2B).

The presence of *E. explanatum* DNA in faeces of animals is of great significance given the need for non-invasive samples for field surveys and surveillance of parasites. In the current study, the efficiency of LAMP-based PCR was initially assessed using live flukes collected from infected liver as a proof of principle. This study has provided a basic design of simplified LAMP-based PCR detection using post mortem samples; however, the same

approach can be tested using faeces samples for surveillance of pathogens in live animals (an ongoing study). Although, extraction of DNA from faeces samples of live animals is a challenging task because liver and rumen flukes of cattle and buffaloes are excreted in hard shell eggs which are impenetrable and laborious to digest. However, recent modifications in conventional protocols have made it feasible.[21,22] The proposed LAMP-based PCR method has many advantages over existing molecular methods for the specific detection of trematode parasite *E. explanatum*. It does not require sophisticated instruments rather it needs a simple dry bath or water bath for the isothermal PCR reaction and results can be recorded by visual observation. Its simplicity and low cost make it more suitable for field-based studies in un-privileged rural areas and low-resourced slaughterhouses.

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Conflict of interest

There are no conflicts of interest of any kind.

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