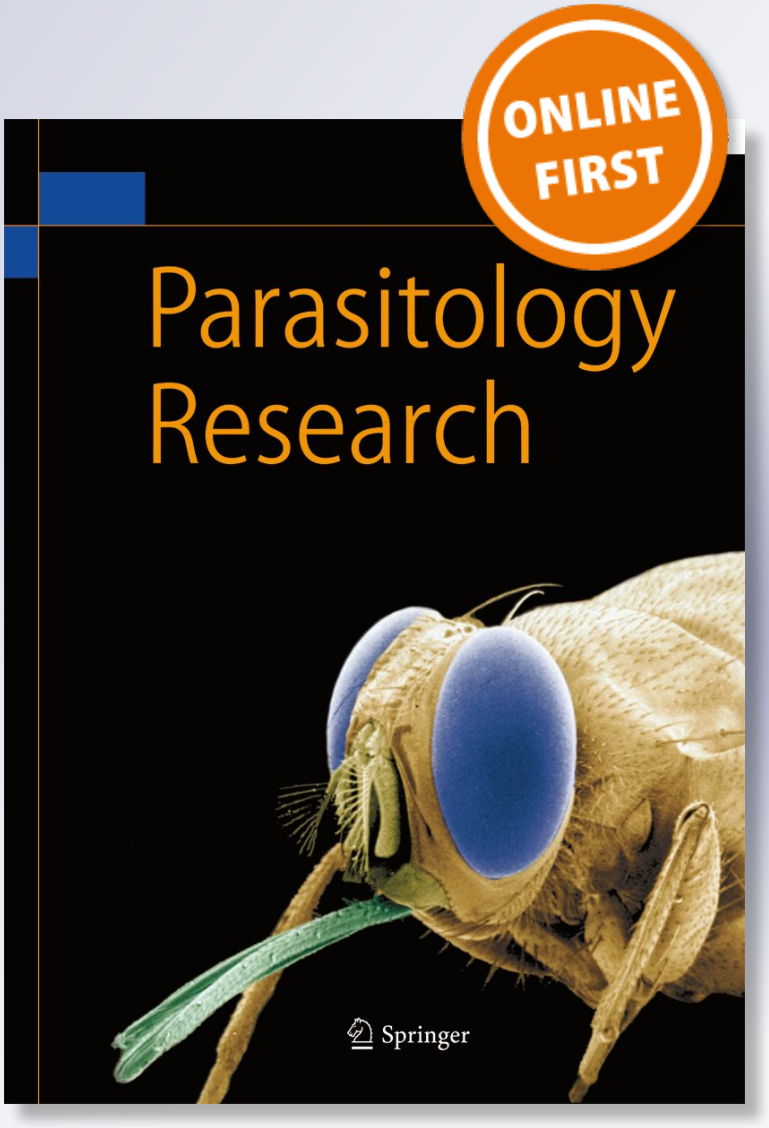
Visual loop-mediated isothermal amplification (LAMP) for the rapid diagnosis of Enterocytozoon hepatopenaei *(EHP) infection*

Sathish Kumar T, Navaneeth Krishnan A, Joseph Sahaya Rajan J, Makesh M, Jithendran K. P, Alavandi S. V & Vijayan K. K

Parasitology Research
Founded as Zeitschrift für Parasitenkunde

ISSN 0932-0113

Parasitol Res
DOI 10.1007/s00436-018-5828-4



Springer

Parasitology Research
https://doi.org/10.1007/s00436-018-5828-4

ORIGINAL PAPER

Visual loop-mediated isothermal amplification (LAMP) for the rapid diagnosis of *Enterocytozoon hepatopenaei* (EHP) infection

Sathish Kumar T[1] · Navaneeth Krishnan A[1] · Joseph Sahaya Rajan J[1] · Makesh M[1] · Jithendran K. P[1] · Alavandi S. V[1] · Vijayan K. K[1]

Received: 10 October 2017 / Accepted: 6 March 2018

Abstract

The emerging microsporidian parasite *Enterocytozoon hepatopenaei* (EHP), the causative agent of hepatopancreatic microsporidiosis, has been widely reported in shrimp-farming countries. EHP infection can be detected by light microscopy observation of spores (1.7 × 1 μm) in stained hepatopancreas (HP) tissue smears, HP tissue sections, and fecal samples. EHP can also be detected by polymerase chain reaction (PCR) targeting the small subunit (SSU) ribosomal RNA (rRNA) gene or the spore wall protein gene (SWP). In this study, a rapid, sensitive, specific, and closed tube visual loop-mediated isothermal amplification (LAMP) protocol combined with FTA cards was developed for the diagnosis of EHP. LAMP primers were designed based on the SSU rRNA gene of EHP. The target sequence of EHP was amplified at constant temperature of 65 °C for 45 min and amplified LAMP products were visually detected in a closed tube system by using SYBR™ green I dye. Detection limit of this LAMP protocol was ten copies. Field and clinical applicability of this assay was evaluated using 162 field samples including 106 HP tissue samples and 56 fecal samples collected from shrimp farms. Out of 162 samples, EHP could be detected in 62 samples (47 HP samples and 15 fecal samples). When compared with SWP-PCR as the gold standard, this EHP LAMP assay had 95.31% sensitivity, 98.98% specificity, and a kappa value of 0.948. This simple, closed tube, clinically evaluated visual LAMP assay has great potential for diagnosing EHP at the farm level, particularly under low-resource circumstances.

Keywords EHP · LAMP · Closed tube LAMP · SYBR green I dye · FTA cards

Abbreviations

EHP	*Enterocytozoon hepatopeanei*
SSU	Small subunit
SWP	Spore wall protein
WFS	White feces syndrome

Introduction

Shrimp aquaculture with varied resources and potential is a very important economic activity and flourishing food production sector in India. Disease outbreaks are one of the major limiting factors frequently challenging the aquaculture sector. Of late, an emerging microsporidian parasite *Enterocytozoon hepatopenaei* (EHP), the causative agent of hepatopancreatic microsporidiosis, has resulted in significant economic losses in many shrimp-farming nations (Rajendran et al. 2016; Tang et al. 2015; Tangprasittipap et al. 2013). Detection of *Enterocytozoon hepatopenaei* (EHP) infection in shrimp by visual inspection of animals is difficult as there are no pathognomonic signs except that it is suspected to be associated with growth retardation and white feces syndrome (WFS) (Rajendran et al. 2016). The target organ for EHP is shrimp hepatopancreas, the power house of the animal, and infection in hepatopancreas may cause impairment in metabolism and ultimately result in stunted growth. EHP infection can be transmitted horizontally through an oral route (cannibalism, predation) (Tang et al. 2016; Tangprasittipap et al. 2013) and possibly by vertical transmission (trans-ovum). So far, no intermediate hosts have been known to be involved.

Early and rapid detection of pathogens is necessary to mitigate the impact of disease outbreak. For the detection of EHP,

Section Editor: Christopher Whipps

✉ Sathish Kumar T
manofpm@gmail.com

[1] ICAR-Central Institute of Brackishwater Aquaculture, #75 Santhome High Road, Raja Annamalai Puram, Chennai 600028, India

Published online: 17 March 2018

polymerase chain reaction (PCR) targeting the small subunit ((SSU)-PCR) ribosomal RNA gene (Tangprasittipap et al. 2013) or the spore wall protein gene ((SWP)-PCR) (Jaroenlak et al. 2016), real-time PCR (Liu et al. 2015), and loop-mediated isothermal amplification (LAMP) (Suebsing et al. 2013, Karthikeyan et al. 2017) have been reported.

Loop-mediated isothermal amplification (LAMP) is an alternative nucleic acid amplification technique based on the principal of strand displacement DNA synthesis and production of stem-loop DNA structures under isothermal conditions (Notomi et al. 2000). Thus, loop-mediated isothermal amplification (LAMP) is highly rapid and specific and does not require any sophisticated equipment. In this study, we demonstrate a very simple, rapid, specific, and closed tube visual LAMP test using SYBR™ green I dye for the detection of EHP.

Materials and methods

Sample collection

Clinical samples including juveniles and sub-adults of *Penaeus vannamei* at 40–91 days of culture (DOC) with an average body weight of 11.0 g (0.5–28.5 g) were collected from shrimp farms located in Andhra Pradesh and Tamil Nadu, India. These farms were experiencing size variation/growth retardation and WFS in shrimp at this stage of farming (Fig. 1). The animals were collected alive and transported to the laboratory on dry ice. Water samples, normal fecal strings, and white fecal strings from the same ponds were also collected and transported on ice. Aseptically dissected hepatopancreas (HP) tissues were smeared on FTA™ elute micro cards (GE Healthcare Whatman, Little Chalfont, UK) at the farm site, dried, and transported to the laboratory at room temperature.

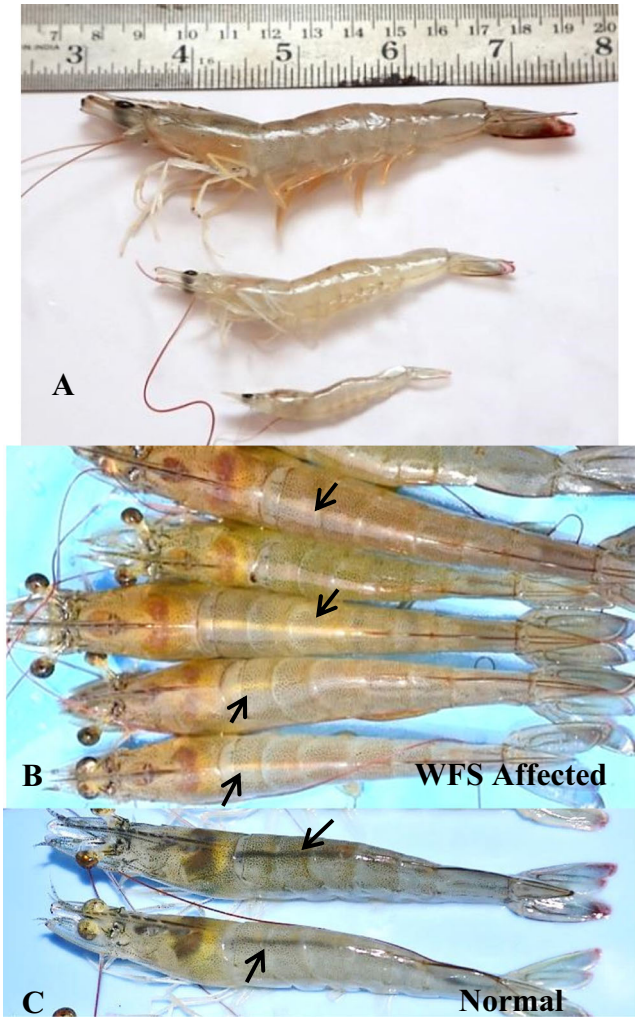


Fig. 1 a Shrimps showing size variation collected from EHP affected ponds, **b** Shrimps affected with white feces syndrome (WFS) showing white gut (arrow) collected from EHP affected ponds, and **c** Shrimps showing normal gut (arrow)

DNA extraction

Genomic DNA was extracted from 106 HP samples and 56 fecal samples using the protocols described earlier (Rajendran et al. 2016) with minor modifications. Briefly, samples were homogenized and digested for 10 min at 95 °C in 500 µl of lysis buffer (50 mM Tris, 1 mM ethylene diamine tetra-acetic acid (EDTA), 500 mM NaCl, 1% SDS, and 0.1 mg proteinase K). The mixture was centrifuged at 12000 rpm (Eppendorf 5810 R, Germany) for 10 min at 4 °C. After centrifugation, the supernatant was collected carefully and two volumes of 100% ethanol were added and kept at – 20 °C for 1 h. The mixture was centrifuged at 12000 rpm for 10 min at 4 °C. The DNA pellet was washed with 70% cold ethanol, air dried, resuspended in nuclease-free water, and stored at – 20 °C. The FTA™ elute micro cards (GE Healthcare Life Sciences, USA) were dried at room temperature in dark, and a small punch (a diameter of ~4 mm) on the FTA card was clipped and the DNA was eluted form the individual punch as per the manufacturer's instructions and stored at – 20 °C until further use.

RNA extraction and cDNA synthesis

RNA was extracted from shrimp samples using TRIzol™ Reagent (Invitrogen, USA) following manufacturer's protocol. The quantity and quality of the extracted RNA was evaluated using a nanospectrophotometer (Implen, Germany) and stored at − 80 °C. Reverse transcription was carried out using iScript cDNA synthesis kit (BioRad, USA) in 10-µl reactions as per the manufacturer's instructions and the cDNA was stored at − 20 °C until further use.

PCR analysis

For the detection of EHP infection in shrimp, PCR amplification was performed using primers targeting the SSU rRNA gene (SSU-PCR) (Tangprasittipap et al. 2013) and the spore wall protein gene ((SWP)-PCR) (Jaroenlak et al. 2016). The PCR amplification was carried out in a 25-μl reaction mixture containing Ampliqon IIII *Taq* DNA Polymerase 2× Mastermix RED (Ampliqon A/S, Denmark) (150 mM Tris-HCl pH 8.5, 40 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2% Tween 20®, 0.4 mM dNTPs, 0.2 units μl^{-1} Ampliqon *Taq* DNA polymerase, Inert red dye and stabilizer), 1.0 μl (10 μM) each of forward primer and reverse primer and 1.0 μl (100 ng) of template DNA. The nested PCR contained the same constituents as that of the first step PCR except for the nested primers and 1.0 μl of the first step product as template. The PCR was carried out on a thermal cycler (Eppendorf, USA) following the reaction conditions as previously described for both SSU-PCR and SWP-PCR. An aliquot of the PCR products was resolved on 2.0% agarose-Tris-acetate-EDTA (TAE) gels containing 0.5 μg ml^{-1} ethidium bromide alongside a 1-kb DNA ladder (SRL Pvt. Ltd., India) and the amplified DNA was visualized under UV illumination using a gel documentation system (Bio-Rad Laboratories, USA).

LAMP primer design and LAMP assay conditions

LAMP primers were designed based on the SSU rRNA gene sequence of EHP (Genbank KF362130) using the Primer explorer version 5 (Table 1). The primer set includes two outer primers and two inner primers targeting four different regions of the SSU rRNA gene sequence of EHP. Two loop primers were designed by visual inspection of the DNA sequence (Table 1). The primers were synthesized as high-performance liquid chromatography (HPLC) grade by Eurofins Scientific Bangalore, India. The LAMP assay was performed in a 25-μl reaction containing 2.5 μl of × 1 Thermopol buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2$ SO_4, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100; pH 8.8 at 25 °C), 1.5 μl of 6 mM $MgSO_4$, 3.5 μl of 10 mM dNTP mix, 1.6 μM of forward inner and backward inner primer each, 0.2 μM of F3 and B3 primers and 0.4 μM of loop forward and loop backward primers, 1 μl of 320 U ml^{-1} of Bst DNA polymerase large fragment (New England Biolabs, USA), and 1 μl of template (100 ng) except for the negative control in which no template was added. The amplification was carried out at different annealing temperature (60, 63, and 65 °C) and different elongation times (30, 45, and 60 min) at 65 °C followed by reaction termination at 80 °C for 5 min in dry bath (Equitron 8550.1.H100, Medica Instruments, India).

Detection of LAMP products

The amplified LAMP products were resolved on agarose gel as described above. For visual detection, 3 μl of 1:10 dilution of SYBR™ green I (× 10,000) (Thermo Fisher Scientific, USA) was directly added into a 24-gauge needle (0.55 × 25 mm/24 × 1), which was plunged into the reaction vials and de ejected with a 1-ml syringe (Dispovan, Hindustan Syringes and Medical Devices, India). Color change in the reaction vials was observed visually and further, the reaction vials were viewed under UV light.

Construction of plasmid DNA

PCR products containing the target sequence obtained following amplification with 779 F and 779 R (Tangprasittipap et al. 2013) were inserted into a pTZ57R/T vector (Thermo Fisher Scientific, USA) and transformed into *Escherichia coli* DH5α competent cells using standard procedures. The plasmid DNA was purified using GenElute™ Plasmid Miniprep Kit (Sigma, Germany) and the concentration was quantified using Nanodrop® (Thermo Fisher Scientific, USA). The plasmid copy numbers were calculated by using the following formula:

$$\text{Copies/ml} = \text{Conc.of Plasmid(g/ml)} \times \text{Avogadro's Constant/Molecular Weight of Plasmid(g/mol)}$$

$$\text{Molecular Weight of Plasmid (g/mol)} = \text{Average Molecular Weight of Bases} \times \text{Total Number of Bases in Plasmid}$$

Evaluation of analytical sensitivity

In order to assess the analytical sensitivity of LAMP, plasmid DNA containing the target sequence was serially diluted tenfold. One microliter of each serially diluted plasmid was isothermally amplified using EHP LAMP primers at 65 °C for 45 min before termination at 80 °C for 5 min. Subsequently, the detection limit of LAMP reaction was determined.

Evaluation of analytical specificity

On the basis of the restriction map of the target sequence, the restriction enzyme *E*co47I (Thermo Fisher Scientific, USA) was selected in order to assess the analytical specificity of LAMP amplified products. Restriction digestion with this enzyme was performed for 2 h at 37 °C and the reaction was terminated by incubation at 65 °C for 20 min. Resultant digest

Table 1 LAMP primers used in this study

Primer name	Sequence (5' to 3')
EHP 2 F3	TGGAGGGCAAGTTTTGGTG
EHP 2 B3	GAGCATCGCTTTCGCCTC
EHP 2 BIP	TGCATCTACGACTACGGACCC/TTTT/GCCGCGGTAATTCCAACTC
EHP 2 FIP	AGTAGCGGAACGGATAGGGAGC/TTTT/CCAGGTGGGGTCTTGAG
EHP 2 LF	ACTGCAGCATCCACCATA
EHP 2 LR	ATGGTATAGGTGGGCAAAGAATG

patterns were compared with online tool LAMP restriction digest fragment analysis. Cross-reactivity of this LAMP protocol was evaluated by testing healthy *P. vannamei* DNA and genomic DNA of crab (*Scylla* sp.), mussel (*Perna* sp.), barnacle (*Valanus* "sp") *Artemia* sp., and shrimp infected with non-targeted pathogens such as white spot syndrome virus (WSSV), infectious hypodermal hematopoietic necrosis virus (IHHNV), and infectious myonecrosis virus (IMNV).

Evaluation of clinical samples

A total of 162 field samples were screened for EHP including 106 HP samples and 56 fecal samples. The 106 HP samples include 40 samples collected on FTA™ elute micro cards and 56 fecal samples (41 normal + 15 WFS affected). The FTA™ elute micro card samples were tested by the EHP LAMP protocol and compared with SWP-PCR. The fecal samples were tested by the EHP LAMP assay and compared with SWP-PCR and also compared with SSU-PCR to check any non-specific amplification with fecal samples (Table 2).

Statistical analysis

Data were analyzed using SISA online statistical software (http://www.quantitativeskills.com/sisa/). The sensitivity and specificity at 95% desired level of confidence and the positive and negative predictive values at 30% prior probability of infection (prevalence) of EHP LAMP assay compared with SWP-PCR were determined. The kappa coefficient (Cohen's kappa coefficient as a measure of agreement for qualitative items) was determined to confirm the consistency of the results between the EHP LAMP assay and SWP-PCR at statistical significance of $P < 0.05$. The obtained kappa values were interpreted as follows (Viera and Garrett 2005): less than a chance agreement (< 0), slight agreement (0.01–0.20), fair agreement (0.21–0.40), moderate agreement (0.41–0.60), substantial agreement (0.61–0.80), almost perfect agreement (0.81–0.99), and perfect agreement (1.00). Thirty percent of prior probability of infection (prevalence) for EHP was chosen based on the surveillance work carried out by ICAR-Central Institute of Brackishwater Aquaculture (unpublished).

Results

LAMP assay conditions

The LAMP amplicons were observed as ladder-like pattern at all annealing temperatures (60, 63, and 65 °C) with the same intensity in agarose gel electrophoresis analysis (Fig. 2). For subsequent testing, 65 °C was chosen as the assay temperature. Assay timings of 30, 45, and 60 min gave LAMP amplicons a ladder-like pattern, but 45 and 60 min gave a ladder-like pattern with higher intensity compared to 30 min (Fig. 3). Thus, the shortest reliable time of 45 min was chosen for the assay time.

Detection of LAMP products

In visual detection, after the addition of SYBR™ green I dye to the reaction vials, the positive LAMP reactions turned green, while the negative LAMP reactions remained orange (Fig. 4a). When SYBR™ green I dye containing LAMP reactions were observed under UV light, fluorescence was observed with the

Table 2 Clinical evaluation with field samples

Clinical samples	No. of samples	SWP-PCR (no. of positive)			LAMP protocol (no. of positive) (this study)
		First step	Nested	Total	
DNA from hepatopancreas*	106	32	17	49	47
DNA from fecal samples**	56	10	5	15	15
Total	162	42	22	64	62

*Includes 40 FTA™ elute micro cards samples; **includes 15 white fecal samples

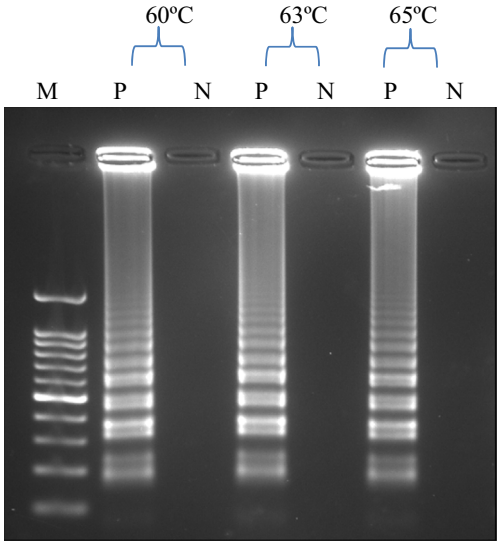


Fig. 2 Gel electrophoresis of EHP LAMP products amplified with different annealing temperatures (60, 63, and 65 °C). M 100-bp marker, P positive, N negative

positive reactions, while the negative reactions did not fluoresce (Fig. 4b). In agarose gel electrophoresis, the positive LAMP reactions produced the ladder-like pattern, whereas negative LAMP reactions produced no bands (Fig. 4c).

Evaluation of analytical sensitivity

The LAMP reaction was tested using tenfold serial dilutions of EHP DNA from purified recombinant plasmids. The detection limit of this LAMP assay was found to be ten copies per reaction vial in 45 min (Fig. 4a–c).

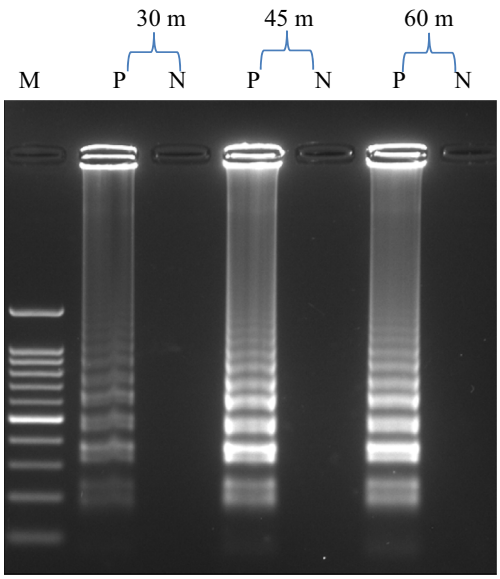


Fig. 3 Gel electrophoresis of EHP LAMP products amplified for different reaction times (30, 45, and 60 min). M 100-bp marker, P positive, N negative

Evaluation of analytical specificity

The analytical specificity of this LAMP protocol was confirmed by the digestion using *E*co47I restriction enzyme. After digestion, the amplified products resulted in a series of bands by agarose gel electrophoresis. The resultant digested products of approximately 205, 170, and 60 bp (Fig. 5) were in accordance with the banding pattern predicted online based on the DNA sequence (http://creisle.github.io/creisle.lamprflp/). This LAMP protocol did not cross-react with healthy shrimp DNA and genomic DNA of crab, mussel, *Artemia*, barnacle, and shrimp infected with non-target pathogens (WSSV, IHHNV, IMNV) (Fig. 6).

Evaluation of clinical samples

A total of 162 field samples including 106 hepatopancreas and 41 normal fecal and 15 white fecal samples were tested for EHP by this LAMP assay and compared with SWP-PCR. Out of the 106 hepatopancreas samples, 47 tested positive for EHP with the LAMP assay and 49 (first step—32, nested—17) were positive for EHP with SWP-PCR. Among 41 normal fecal samples, seven were positive with nested PCR for SSU-PCR and none of the samples tested positive for EHP by LAMP assay and SWP-PCR. All of the 15 white fecal samples were positive for EHP by the LAMP assay, SSU-PCR, and SWP-PCR (10 first step, 5 nested). Out of the 40 samples collected in FTA™ elute micro cards of a single punch (~4 mm), 17 samples were detected with EHP by both this LAMP assay and SWP-PCR (first step—11, nested—6).

Statistical analysis

At a desired level of confidence (95%), the sensitivity and specificity values of this EHP LAMP assay were calculated as 95.31% (CI 86.91–99.02%) and 98.98% (CI 94.45–99.97%), respectively. Then, at 30% of prior level of probability of EHP infection (prevalence), the positive predictive value and the negative predictive value were calculated as 97.56 and 98.06%, respectively. The agreement between EHP LAMP assay and SWP-PCR was almost in perfect agreement with kappa value of 0.948 at a statistical significance of $P < 0.05$.

Discussion

EHP has emerged as a serious threat to shrimp aquaculture worldwide. EHP infections in farmed shrimp do not cause mass mortality, but inflict significant economic loss due to stunted growth and reduced feed consumption. Thus, rapid diagnosis of EHP at early stages is very important for intervention and implementation of preventive measures at the

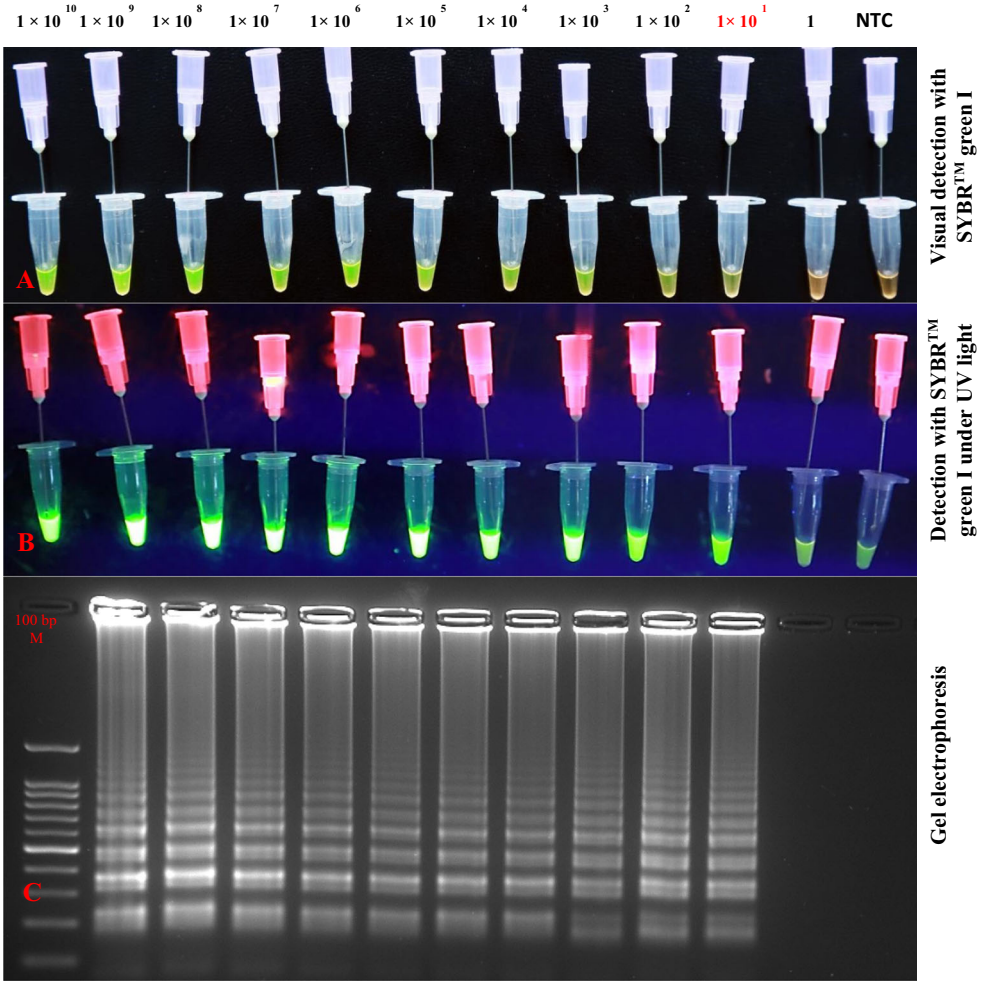


Fig. 4 **a** Visual detection of LAMP products serially diluted EHP plasmid DNA with SYBR™ green. **b** Detection of serially diluted EHP plasmid DNA-LAMP products with SYBR™ green under UV light, **c** Gel electrophoresis of serially diluted EHP plasmid DNA-LAMP products

farm level. In this study, we describe a simple, rapid, sensitive, and specific diagnostic tool for diagnosis of EHP at the farm level using a visual LAMP assay.

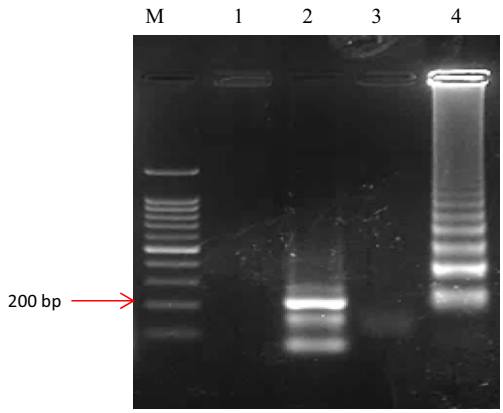


Fig. 5 Gel electrophoresis of amplified EHP LAMP products digested with restriction enzyme *Eco*47I and undigested amplified LAMP product. M—100-bp marker, 1—negative control digested with restriction enzyme *Eco*47I, 2—EHP LAMP product digested with restriction enzyme *Eco*47I, 3—negative control and 4- Undigested EHP lamp product

A LAMP assay is a simple diagnostic protocol with numerous advantages such as high specificity and sensitivity, the capacity to work at isothermal conditions, field adaptability, and no requirement for sophisticated equipment (Notomi et al. 2000, Tomita et al. 2008). However, the LAMP assay occasionally suffers from product cross-contamination (Angamuthu et al. 2012 and Lau et al. 2010). The opening of the reaction vial after amplification often results in aerosol contamination (Lau et al. 2010). A few closed tube methods were described earlier (Hong et al. 2012, Karthik et al. 2014 and Liang et al. 2013). During the study, we often encountered false-positive results and cross-contamination after opening the reaction lid. It was observed that opening the vial in the fume hood for adding the dye did not cause cross-contamination, but again, the use of a fume hood at farm level had its own limits. Thus, to overcome such aerosol contamination, in this study, a simple and cheap closed tube method was developed by using a syringe and a needle to add SYBR™ green I dye in to the vial after amplification. For visual detection of LAMP products, metal indicator dyes such as hydroxy napthol blue, malachite green, and SYBR™ green I dye were used. Although all the dyes produced very clear and visually

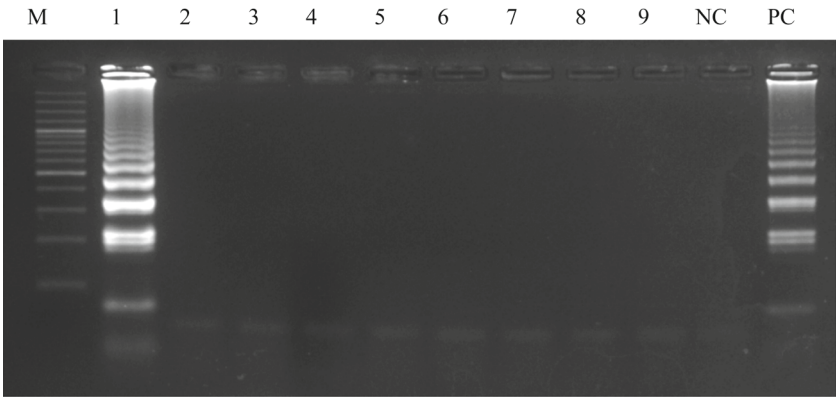


Fig. 6 Assessment of cross-reactivity of the EHP LAMP assay. M 100-bp marker, 1 EHP DNA, 2 healthy shrimp DNA, 3 crab DNA, 4 mussel DNA, 5 *Artemia* DNA, 6 barnacle, 7 WSSV-infected DNA, 8 IHHNV-infected DNA, 9 IMNV-infected cDNA, NC negative control, PC positive control

contrasting negative and positive results, except SYBR™ green I, other dyes had issues with sensitivity and reproducibility (Karthik et al. 2014, Tanner et al. 2015). In addition to good sensitivity and visual clarity, SYBR™ green can also fluoresce in the presence of UV light, which can be viewed with a simple handheld UV light at farms.

Previously, few LAMP protocols were described for the detection of EHP. The visual LAMP protocol described by Suebsing et al. (2013) employed a gold nanoparticle (AuNP) probe, which was added after the amplification and hence required the opening of tubes. The recently described LAMP assay by Karthikeyan et al. (2017) relied on turbidity that must be measured by a Loopamp Realtime Turbidimeter. The visual LAMP assay developed in this study is a closed tube assay which requires a simple dry bath, wherein the issues of contamination are avoided and simpler to use than the previously described protocols.

In the absence of gold standard, this EHP LAMP assay results were compared with SWP-PCR. Out of the 162 clinical samples, EHP was detected in 62 samples by LAMP and in 64 samples by SWP-PCR. Four samples showed discordant results between this LAMP assay and SWP-PCR. Three of these samples by SWP-PCR nested positive but only partially detected with LAMP assay, were defined positive in one run, and were found negative in the replicate. Additional four runs were performed for those three samples by LAMP assay; out of three samples, two were found positive in three runs and negative in one run and one sample found positive in two runs and negative in the other two runs. Further sequencing results of those three samples revealed the false-negative amplification in the few runs of LAMP assay. Subsequently, one sample was found negative by SWP-PCR but positive by LAMP assay. This may be due to the presence of inhibitors in the sample which may inhibit the PCR amplification (Nasarudin et al. 2015). Further, that sample LAMP amplicon was confirmed for EHP amplification by restriction enzyme analysis with *E*co47I. Inconsistencies in results were mainly obtained when samples with low copies of EHP were tested. Further, this LAMP protocol has not resulted in any non-specific amplification in the 41 fecal samples when compared with SSU-PCR and did not cross-react with genomic DNA of crab, mussel, *Artemia*, barnacle, and healthy shrimp and with aquatic viral pathogens such as WSSV, IHHNV, and IMNV. In comparison with SWP-PCR as reference test, at the 95% confidence limit, the sensitivity and specificity values of this EHP LAMP assay were 95.31 and 98.98%, respectively, and at 30% prior level of probability or prevalence of EHP infection, the positive predictive value (probability of animals infected with EHP when the LAMP assay is positive) was 97.56% and the negative predictive value (probability of animals not infected with EHP when the LAMP assay is negative) was 98.01%.

When the conventional DNA extraction protocol was compared with FTA™ elute micro card-eluted DNA (with 40 samples) for the detection of EHP, there was no difference in the outcome of both LAMP assay and SWP-PCR. For DNA extraction from EHP spores, phenol/chloroform method (Sambrook et al. 1989), lysis buffer method (Rajendran et al. 2016), glass beads (for breaking spore wall) combined with lysis buffer method, glass beads combined with FTA cards (Yan et al. 2014), and FTA cards alone were used initially in this study. During the course of the study, we found that the samples collected on the FTA cards yielded DNA quality equivalent to other methods. The FTA cards are reported to be impregnated with chemicals that can lyse cells, denature proteins, and protect nucleic acids from nucleases, oxidation, and UV damage (Yan et al. 2014). The use of FTA filter paper for DNA extraction of other microsporidians such as *Enterocytozoon bieneusi*, *Encephalitozoon cuniculi*, and *E. intestinalis* has earlier been described (Subrungruang et al. 2004, Ndzi et al. 2016). With FTA cards, the EHP DNA can be preserved for subsequent amplification and can be safely transported at room temperature for subsequent testing and the same was verified with *E. bieneusi* and other microsporidians (Ndzi et al. 2016).

In this study, we demonstrated an accurate, sensitive visual detection of EHP using the SYBR™ green LAMP assay. This visual EHP LAMP assay could consistently detect EHP at as

low as ten copies within 45 min. The detection limit of this EHP LAMP assay was equivalent to the nested SWP-PCR and SSU-PCR and this LAMP assay also showed almost perfect agreement with SWP-PCR (kappa value of 0.94). This assay was found to be compatible with DNA eluted from the FTA™ elute micro cards without any loss of sensitivity. This EHP LAMP assay is a simple, closed tube, and visual protocol evaluated against field samples for the diagnosis of EHP among the reported EHP LAMP protocols. This visual LAMP method can be possibly performed at the farm level under low-resource circumstances without the need of expensive equipment and DNA extraction procedures by combining with FTA cards and a simple dry bath. In conclusion, the present diagnostic tool would be useful to intervene the EHP epizootics, rapidly at early stages and would be of help to study the life cycle, vertical transmission, involvement of intermediate hosts if any, and underlying transmission mechanism of this microsporidian to combat hepatopancreatic microsporidiosis in shrimp hatcheries and grow-out ponds.

Acknowledgements The authors acknowledge the Indian Council of Agricultural Research (ICAR) Consortia Research Platform on Vaccines and Diagnostics, Govt. of India, for the financial support to carry out this work. The authors also thank the shrimp farmers of Tamil Nadu and Andhra Pradesh for providing samples and information for this study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Angamuthu R, Baskaran S, Gopal DR, Devarajan J, Kathaperumal K (2012) Rapid detection of the Marek's disease viral genome in chicken feathers by loop-mediated isothermal amplification. J Clin Microbiol 50(3):961–965

Hong M, Zha L, Fu W, Zou M, Li W, Xu D (2012) A modified visual loop-mediated isothermal amplification method for diagnosis and differentiation of main pathogens from Mycobacterium tuberculosis complex. World J Microbiol Biotechnol 28(2):523–531

Jaroenlak P, Sanguanrut P, Williams BAP, Stentiford GD, Flegel TW, Sritunyalucksana K, Itsathitphaisarn O (2016) A nested PCR assay to avoid false positive detection of the microsporidian *Enterocytozoon hepatopenaei* (EHP) in environmental samples in shrimp farms. PLoS One 11(11):e0166320. https://doi.org/10.1371/journal.pone.0166320

Karthik K, Rathore R, Thomas P, Arun TR, Viswas KN, Dhama K, Agarwal RK (2014) New closed tube loop mediated isothermal amplification assay for prevention of product cross-contamination. Methods X 1:137–143

Karthikeyan K, Sharma A, Mekata T, Itami T, Sudhakaran R (2017) Rapid and sensitive real-time loop-meditated isothermal amplification for the detection of *Enterocytozoon hepatopenaei* of shrimp. Aquaculture 481:119–123. https://doi.org/10.1016/j.aquaculture.2017.08.036

LAMP restriction digest fragment analysis http://creisle.github.io/creisle.lamprflp/

Lau YL, Meganathan P, Sonaimuthu P, Thiruvengadam G, Nissapatorn V, Chen Y (2010) Specific, sensitive, and rapid diagnosis of active toxoplasmosis by a loop-mediated isothermal amplification method using blood samples from patients. J Clin Microbiol 48(10):3698–3702

Liang C, Cheng S, Chu Y, Wu H, Zou B, Huang H, Xi T, Zhou G (2013) A closed-tube detection of loop-mediated isothermal amplification (LAMP) products using a wax-sealed fluorescent intercalator. J Nanosci Nanotechnol 13(6):3999–4005

Liu Z, Zhang QL, Wan XY., Huang J (2015). Development of real-time PCR assay for detection of microsporidian *Enterocytozoon hepatopenaei* and detection in shrimp samples under different growth rates. Fish. Sci. (in Press Chinese, English Abstr)

Nasarudin SNSA, Zainudin NS, Bernadus M, Nawi AM, Hanafiah A, Osman E (2015) Loop-mediated isothermal amplification for rapid molecular detection of *Enterocytozoon bieneusi* in faecal specimens. J Med Microbiol 64(11):1329–1334

Ndzi ES, Asonganyi T, Nkinin MB, Xiao L, Didier ES, Bowers LC, Nkinin SW, Kaneshiro ES (2016) Fast technology analysis enables identification of species and genotypes of latent microsporidia infections in healthy native Cameroonians. J Eukaryot Microbiol 63(2):146–152

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28(12):E63

Rajendran KV, Shivam S, Ezhil Praveena P, Joseph Sahaya Rajan J, Sathish Kumar T, Avunje S, Jagadeesan V, Prasad Babu SVANV, Pande A, Navaneeth Krishnan A, Alavandi SV, Vijayan KK (2016) Emergence of *Enterocytozoon hepatopenae*i (EHP) in farmed *Penaeus (Litopenaeus) vannamei* in India. Aquaculture 454:272–280. https://doi.org/10.1016/j.aquaculture.2015.12.034

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual (No. Ed. 2). Cold spring harbor laboratory press

SISA online statistical software www.quantitativeskills.com/sisa/

Subrungruang I, Mungthin M, Chavalitshewinkoon-Petmitr P, Rangsin R, Naaglor T, Leelayoova S (2004) Evaluation of DNA extraction and PCR methods for detection of *Enterocytozoon bienuesi* in stool specimens. J Clin Microbiol 42(8):3490–3494

Suebsing R, Prombun P, Srisala J, Kiatpathomchai W (2013) Loop-mediated isothermal amplification combined with colorimetric nanogold for detection of the microsporidian *Enterocytozoon hepatopenaei* in penaeid shrimp. J Appl Microbiol 114(5):1254–1263. https://doi.org/10.1111/jam.12160

Tang KFJ, Pantoja CR, Redman RM, Han JE, Tran LH, Lightner DV (2015) Development of in situ hybridization and PCR assays for the detection of *Enterocytozoon hepatopenaei* (EHP), a microsporidian parasite infecting penaeid shrimp. J Inverteb Pathol 130:37–41. https://doi.org/10.1016/j.jip.2015.06.009

Tang KFJ, Han JE, Aranguren LF, White-Noble B, Schmidt MM, Piamsomboon P, Risdiana E, Hanggono B (2016) Dense populations of the microsporidian *Enterocytozoon hepatopenaei* (EHP) in feces of *Penaeus vannamei* exhibiting white feces syndrome and pathways of their transmission to healthy shrimp. J Invertebr Pathol 140:1–7. https://doi.org/10.1016/j.jip.2016.08.004

Tangprasittipap A, Srisala J, Chouwdee S, Somboon M, Chuchird N, Limsuwan C, Srisuvan T, Flegel TW, Sritunyalucksana K (2013) The microsporidian *Enterocytozoon hepatopenaei* is not the cause of white feces syndrome in whiteleg shrimp *Penaeus (Litopenaeus) vannamei*. BMC Vet Res 9(1):139. https://doi.org/10.1186/1746-6148-9-139

Tanner NA, Zhang Y, Evans TC Jr (2015) Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. BioTechniques 58:59–68

Tomita N, Mori Y, Kanda H, Notomi T (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat Protoc 3(5):877–882

Viera AJ, Garrett JM (2005) Understanding interobserver agreement: the kappa statistic. Fam Med 37(5):360–363

Yan W, Shen Z, Tang X, Xu L, Li Q, Yue Y, Xiao S, Fu X (2014) Detection of *Nosema bombycis* by FTA cards and loop-mediated isothermal amplification (LAMP). Curr Microbiol 69(4):532–540