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Molecular diagnostic methods for detection of *Thelohania contejeani* (Microsporidia), the causative agent of porcelain disease in crayfish

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ABSTRACT: Diagnosis of *Thelohania contejeani* in the crayfish *Astacus astacus* is currently based on observation of gross clinical signs — opaque appearance of the abdomen and whitish colouration of the musculature — and confirmed by microscopic examination of histological sections of muscle. We have developed 2 molecular diagnostic methods for sensitive and rapid detection of porcelain disease in its early stages: PCR and loop-mediated isothermal amplification (LAMP). The PCR test utilises a primer based on the *T. contejeani* small subunit ssu ribosomal RNA (ssu rRNA) gene and amplified parasite DNA with high specificity and a detection limit of 10^{-5} dilution. The LAMP assay involves incubation of the target DNA with a set of 6 primers and *Bst* DNA polymerase for 60 min at 65°C in a water bath or heating block, followed by visualisation of the reaction products with the SYBR Green I stain; sensitivity of visual detection with SYBR Green I is equivalent to that with agarose gel electrophoresis. The LAMP assay can detect *T. contejeani* DNA to a dilution of 10^{-7}. The LAMP assay is 100 times more sensitive than the PCR test and is the method we recommend as an alternative to traditional means of diagnosing *T. contejeani.*

KEY WORDS: *Thelohania contejeani* · PCR · Loop-mediated isothermal amplification · Diagnosis · Crayfish

INTRODUCTION

Thelohaniasis, or porcelain disease, is a significant disease, which affects a number of decapod crustaceans, including freshwater crayfish. It is caused by the microsporidian parasite *Thelohania contejeani* (Sprague & Couch 1971), and was originally described in Europe by Henneguy & Thelohan (1892) as the causative agent of a disease which resulted in heavy losses in crayfish populations. *T. contejeani* infects 2 European crayfish species, *Astacus astacus* (Schäperclaus 1954, Sumari & Westman 1969, Voronin 1971) and *Austropotamobius pallipes* (Dollfus 1935, Pixell Goodrich 1956, Vey & Vago 1973). The disease has also been reported in several crayfish species from different countries (McGriff & Modin 1983).

According to Kudo (1924) and Bykhovskaya-Pavlovskaya et al. (1964), crayfish become infected through ingestion of *Thelohania contejeani* spores. The amoeboid germ of the spore then emerges, penetrates the gut, and reaches the muscle tissue via the haemocoele. Asexual reproduction takes place in the muscle, which leads to a dramatic increase in parasite cells. Finally, pansporoblasts develop and transform directly into sporoblasts containing 8 spores, which can be released upon rupture of the sporoblast membrane. Lom et al. (2001) reported that *T. contejeani* has 2 simultaneous routes of sporogony: either diplokaryotic sporonts produce 8 uninucleated spores with 9 to 10 turns of the polar tube within a sporophorous vesicle wall, or single diplokaryotic sporonts produce small membrane-bound compartments, in which they transform into mature diplokaryotic spores with 5 to 6 turns of the polar tube.

Thelohania contejeani infects both juvenile and adult crayfish of either sex. Infection causes the abdominal muscles to appear opaque and milky due to spore production, as opposed to healthy muscle which is grey and

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translucent. With advanced infection, the crayfish appear sluggish and the tail-flick escape reaction is incompetent (Cossins & Bowler 1974, Alderman & Polglase 1988). *T. contejeani* can cause considerable mortality in both natural and artificially reared crayfish populations (Henneguy & Thelohan 1892, Vey & Vago 1972).

Diagnosis of porcelain disease has traditionally relied on observation of gross clinical signs — the white musculature and opaque abdomen — followed by observation of pansporoblasts (5 µm diameter) in histological muscle sections (Bower et al. 1994). Nevertheless, in early stages of the disease, clinical signs can be either difficult to observe, or entirely absent (Bower et al. 1994); hence, there is a great demand for a rapid, sensitive and specific technique for diagnosis of *Thelohania contejeani* to protect crayfish populations by restricting spread of the disease.

DNA-based detection methods are increasingly being developed for diagnosis of pathogens. One molecular method, polymerase chain reaction (PCR), is now a standard technique in molecular biology for amplification and identification of specific DNA fragments. A second, novel DNA–based diagnostic approach, loop-mediated isothermal amplification (LAMP), was originally developed by Notomi et al. (2000) for rapid amplification of DNA with high specificity and efficiency under isothermal conditions. LAMP can amplify a few copies of DNA to 10^9 copies in <1 h under isothermal conditions (Thekisoe et al. 2005). In addition, a LAMP reaction can be easily determined positive by the naked eye, without agarose gel electrophoresis (Mori et al. 2001, Iwamoto et al. 2003, El-Matbouli & Soliman 2005a,b, Soliman & El-Matbouli 2005).

The aim of this work was to develop and compare advanced, sensitive, specific and rapid diagnostic assays to detect presporogonic stages of Thelohania *contejeani* in crayfish using PCR and LAMP methods.

MATERIALS AND METHODS

Isolation of *Thelohania contejeani* spores and DNA extraction. In the present study we used 17 freshwater crayfish naturally infected with *Thelohania contejeani*, and 5 uninfected indidviduals from the same population. Highly infected abdominal muscle was dissected out of the diseased crayfish and pulped with a scalpel. The resultant spore suspension was pressed through a 10 µm filter, using a pestle, to separate spores from muscle tissue. The spores were then washed several times with double-distilled water through repeated centrifugation at 4000 × *g* for 2 min.

DNA was extracted directly from infected crayfish muscle tissues and from separated *Thelohania contejeani* spores using a QIAamp DNA mini kit (QIAGEN). Infected tissue was thoroughly homogenised in liquid nitrogen, and 20 mg of tissue powder was then transferred to a 2 ml microcentrifuge tube, also in liquid nitrogen. We added 180 µl of lysis buffer and 20 µl of Proteinase K to the powder followed by incubation at 56°C for 1 to 3 h. DNA was then extracted according to the manufacturer's instructions, eluted with nanopure water, and stored at –20°C.

Oligonucleotides. A set of 6 primers was designed for LAMP based on the *Thelohania contejeani* small subunit ribosomal RNA gene (ssu rDNA) (GenBank Accession No. AF 492593). This set of primers consisted of 2 outer primers (F3 and B3), 2 inner primers (forward inner primer FIP, and reverse inner primer, BIP) and 2 loop primers (LoopF and LoopB).

FIP contained the complementary sequence of F1 primer (24 nt), TTTT linker and sense sequence of F2 primer (22 nt): 5′-CCTTAGACCTAGTAGCCATCT CTCTTTTTACGTAGGCGATGACGGGTAAC-3′; BIP contained the sense sequence of B1 (21 nt), TTTT linker and the antisense sequence of B2 (20 nt): 5′-AGC AGGCGCGAAACTTACCCCTTTTGGCTGC TGGCACCAGACTTG-3′. F3 primer (22 nt): 5′-AGC-TAGTATGTAGG GTAA GGGC-3′; and B3 primers (22 nt): 5′-ACTCTT GGAGC TGGAATTACCG-3′.

To accelerate the LAMP reaction, 2 loop primers were constructed: LoopF (22 nt) 5′-CCGGAATA-GAACCCTAATTCCC-3′, and LoopB (28 nt) 5′-TGC-TATTTAGTAGTGAGGTAGTTATAAG-3′. The location of the LAMP primers on the DNA sequence is illustrated in Fig. 1. The LAMP outer forward primer F3 and the reverse primer B3 were used for the standard PCR reaction to amplify a 261 bp fragment.

Fig. 1. *Thelohania contejeani.* (GenBank Accession No. AF 492593) Partial ssu rDNA sequence used to construct the inner and outer primers (in italics). Inner primers FIP and BIP comprise complementary sequence to F1 and sense sequence of F2, and sense sequence of B1 and complementary sequence of B2, respectively

LAMP reaction. The LAMP reaction for detection of *Thelohania contejeani* was carried out in 25 µl final volume comprising 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 5 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton x-100, 1.6 M betaine, deoxynucleotide triphosphates (2.8 mM each), 1.6 µM each of FIP and BIP, 0.8 µM each of loop-F and loop-B, 0.2 µM each of F3 and B3 primers, 8U *Bst* DNA polymerase (New England BioLabs), 2 µl template DNA and 25 µl distilled water. DNA template was omitted from the negative control reaction. The mixture was incubated at 65°C for different periods (10 to 60 min, in 5 min increments). After incubation, the reaction was terminated by heating at 80°C for 2 min. Different concentrations and combinations of the primers FIP, BIP, F3 and B3 were tested with and without the F and B loop primers.

Detection of LAMP products. For visual detection, 1 µl of 1:10 diluted SYBR Green I nucleic acid gel stain, 10 000 × concentration in DMSO (Cambrex Bio Science) was added to the reaction mixture and any colour change noted.

For agarose gel electrophoresis, 2 µl of the reaction mixture was loaded on an ethidium bromide (1 mg ml^{-1}) -stained 2% agarose gel, electrophoresed, and then photographed under UV light. A 100 bp DNA ladder (Cambrex Bio Science) was used as a molecular weight marker.

PCR. PCR was performed in a 50 µl reaction mixture containing 46 µl 1.1 × ReadyMix PCR Master mix (AB Gene) comprising 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween 20, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 1.25 U *Taq* DNA polymerase and red dye for electrophoresis, and 4 µl template DNA with forward and reverse primers (F3 and B3). A gradient PCR was conducted to determine optimal primer annealing temperature, and different concentrations of forward and reverse primers were tested.

The PCR products were analysed on a 1.5% agarose gel, stained with ethidium bromide, and then visualised under UV light. A 100 bp DNA ladder (Cambrex Bio Science) was used as molecular weight marker.

Cloning and sequencing of *Thelohania contejeani* PCR products. For sequencing, DNA fragments (261 bp) were separated from excess primers and unincorporated nucleotides using the MinElute gel extraction kit (QIAGEN), and then cloned into the pCR® 4-TOPO® vector using a TOPO TA cloning® kit for sequencing (Invitrogen), as per the manufacturer's instructions. Recombinant plasmids were purified from *E. coli* using a Fast plasmid mini kit (Eppendorf AG), following the manufacturer's instructions. Plasmid concentrations were determined by a spectrophotometer (Eppendorf biophotometer). Cloned PCR products were sequenced in a commercial sequencing laboratory (SEQLAB), and then compared with the type *T. contejeani* ssu rRNA gene sequence.

Specificity of LAMP and PCR reactions. The specificity of the LAMP and PCR assays for sole amplification of *Thelohania contejeani* was appraised by testing it against DNA from other *Thelohania* species — *T. montirivulorum*, *T. solenopsae* and non-infected crayfish muscle.

Sensitivity of LAMP and PCR assays. The sensitivity of the LAMP and PCR assays was compared using 10-fold serial dilutions of *Thelohania contejeani* template DNA to determine the lower detection limit of each test.

RESULTS

Optimisation of reaction conditions for detection of *Thelohania contejeani* by LAMP

Preliminary optimisation of the LAMP assay was carried out using Primers FIP, BIP, F3 and B3. The reaction mix was incubated at temperatures from 60 to 65°C for

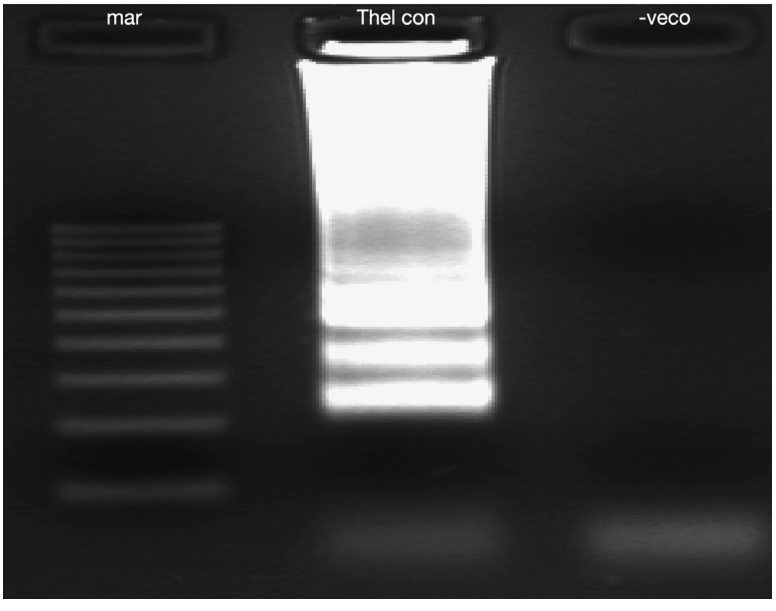

Fig. 2. *Thelohania contejeani.* Agarose gel showing LAMP amplification products of *T. contejeani* DNA, which appears as multiple bands of different molecular weights. Lanes: mar = 100 bp DNA molecular weight standard; Thel con = *T. contejeani* products using Primers FIP, BIP, F3, B3, LoopF, LoopB; -veco = no template negative control

60 min; the optimum temperature of the LAMP assay was 65°C, at which temperature bands were especially clear (Fig. 2). The optimal reaction time, using the 4 primers, was 45 min at 65°C for detection of amplification products (data not shown). Addition of the 2 loop primers, F and B, resulted in amplification products which were detected as early as 25 min at 65°C (Fig. 3). On the gel, the LAMP products appeared as multiple bands of different molecular weight up to the loading well (Fig. 2). The sensitivity of detection of the products visually, with SYBR Green I, was equivalent to that of agarose gel electrophoresis; addition of SYBR Green I to the reaction tubes produced a green colour for positive samples while the negative control samples remained orange (Fig. 4). In summary, the LAMP assay was optimised to detect *Thelohania contejeani* with high sensitivity by incubation with all 6 primers at 65°C for 60 min.

Optimisation of reaction conditions for detection of *Thelohania contejeani* by PCR

The best amplification of the specific 261 bp DNA fragment (Fig. 5) was achieve under the following conditions: 10 pmol from each primer (F3 and B3) with cycling conditions comprising initial denaturation at 96°C for 5 min, followed by 38 cycles of denaturation at 95°C for 45 s, primer annealing at 66°C for 45 s, and extension at 72°C for 45 s, with a final elongation step at 72°C for 5 min.

Cloning and sequencing of the 261 bp PCR product revealed 100% homology with the ssu rRNA gene of *Thelohania contejeani* in GenBank.

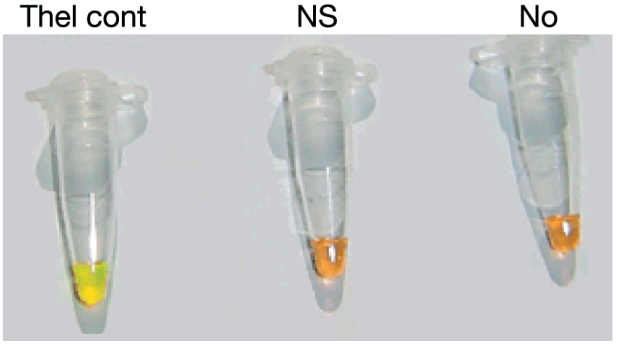

Fig. 4. *Thelohania contejeani.* Appearance of LAMP products visualised with SYBR Green I stain. Thel cont = reaction positive for *T. contejeani*, green colouration; NS = negative reaction, orange colouration; NO = no template (negative control)

Specificity of LAMP and PCR assays to *Thelohania contejeani*

Both the LAMP and PCR assays amplified *Thelohania contejeani* DNA only, with no reaction to *T. montirivulorum*, *T. solenopsae*, un-infected crayfish or the no-template negative control (Figs. 6 & 7).

Sensitivity of LAMP and PCR assays

A 10-fold serial dilution of *Thelohania contejeani* DNA revealed the detection limits of the reactions (Fig. 8): the LAMP assay detected down to a dilution of 10^{-7} while the PCR detected only to a dilution of 10^{-5}.

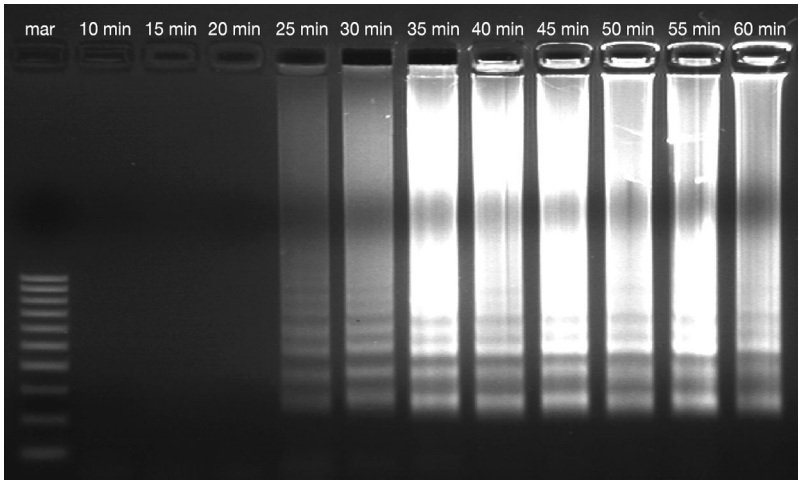

Fig. 3. *Thelohania contejeani.* Agarose gel showing effect of varying amplification time in *T. contejeani*–LAMP reaction. Amplification using Primers FIP, BIP, F3, B3, LoopF and LoopB was carried out at 65°C for time periods indicated. mar = 100 bp DNA molecular weight standard. *T. contejeani* DNA was detectedable as early as 25 min

DISCUSSION

Crayfish are of ecological and economic importance, as their flesh is edible and large quantities are consumed in many countries. Freshwater crayfish farming is expanding around the world. The microsporidian parasite *Thelohania contejeani* has impacted crayfish populations and caused economic losses to the industry. The parasite infects the crayfish musculature, with no visible symptoms of disease until infection has progressed to the point where the abdominal muscle tissue is white and opaque. These physical signs only appear during the final stages of the disease, and are rapidly followed by loss of muscle function and death. Hence, early recognition of the disease and its subsequent eradication

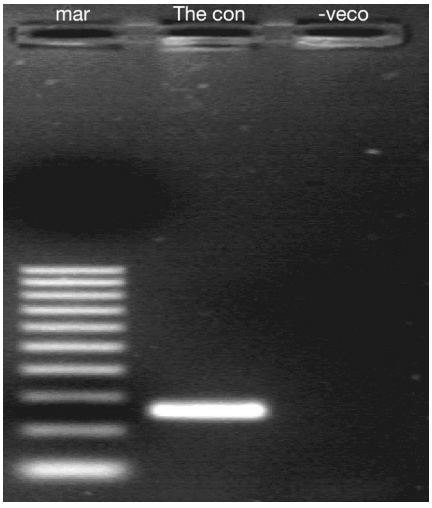

Fig. 5. *Thelohania contejeani.* Agarose gel showing PCR product of DNA. The primers used, F3 and B3, amplified the 261 bp DNA fragment of *T. contejeani*, while there was no amplification in the negative control. Lanes: mar = 100 bp DNA molecular weight standard; The con = *T. contejeani* DNA; -veco = negative control

is essential in maintaining a viable and productive farm (Cossins 1973). Given a lack of methods for diagnosing porcelain disease in its early stages, we have developed 2 molecular assays for sensitive detection of *T. contejeani*.

Molecular techniques provide rapid and sensitive ways to diagnose disease, and often overcome the shortcomings of traditional diagnostic methods. PCR assays facilitate diagnosis by direct visualisation of specific bands on ethidium bromide-stained gels; this is easier and faster than processing samples for histological examination. We have developed a PCR assay for diagnosis of *Thelohania contejeani* which employs forward and reverse primers constructed from the ssu rRNA gene. The assay was optimised with respect to primer concentration, annealing temperature and other parameters for detection of *T. contejeani* DNA with high specificity and sensitivity. The assay does not detect DNA of other closely related *Thelohania* species, and can detect *T. contejeani* DNA to a dilution of 10^{-5}; it is more highly sensitive than traditional methods of diagnosing porcelain disease.

A novel molecular diagnostic technique was also developed for isothermal amplification of *Thelohania contejeani* DNA by LAMP assay. LAMP assays have been used previously for rapid diagnosis of the causative agents of different diseases which affect aquaculture (Caipang et al. 2004, Gunimaladevi et al. 2004, 2005, Kono et al. 2004, Savan et al. 2004, El-Matbouli & Soliman 2005a,b, Soliman & El-Matbouli 2005). Our LAMP assay was optimised to amplify *T. contejeani* DNA using a set of 6 primers incubated at 65°C for 60 min in the presence of a *Bst* DNA polymerase enzyme. Although the LAMP assay required only 4 primers, 2 outer and 2 inner, that recognise 6 different regions of the target sequence (Notomi et al. 2000), addition of 2 loop primers was found to enhance and accelerate the reaction, and increase not only the sensitivity but also the specificity of the assay. The 6 primers recognised 8 distinct regions of the target DNA, which minimised the probability of false positive results (Nagamine et al. 2002, Maeda et al. 2005). Although the LAMP assay could detect *T. contejeani*

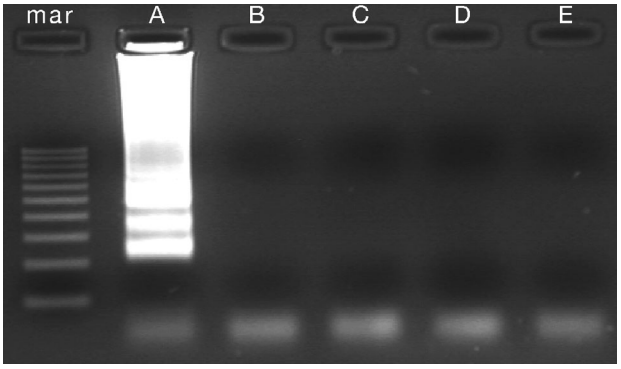

Fig. 6. *Thelohania contejeani*. Agarose gel electrophoresis, demonstrating specificity of LAMP for *T. contejeani*. LAMP reaction was carried out at 65°C for 1 h using a set of 6 primers. Lanes: mar = 100 bp molecular weight marker; A = amplification products of *T. contejeani* DNA; B = no amplification of *T. montirivulorum;* C = no amplification of *T. solenopsae;* D = no amplification of uninfected crayfish DNA; E = no amplification of no-template control

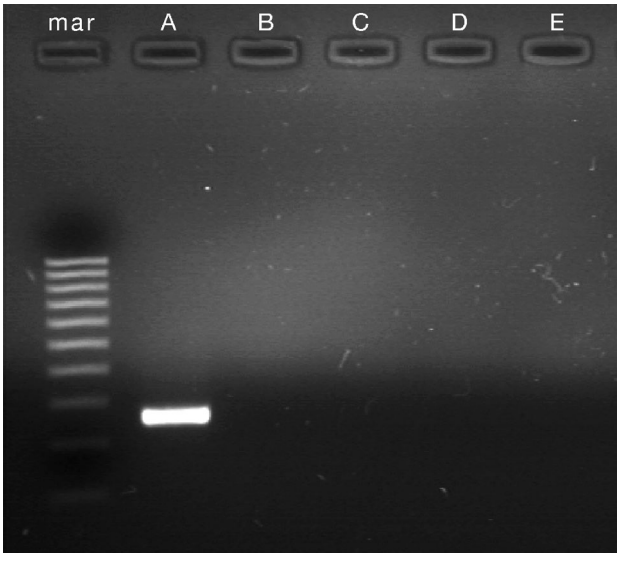

Fig 7. *Thelohania contejeani.* Agarose gel electrophoresis demonstrating specificity of PCR for *T. contejeani.* Other details as in Fig. 6

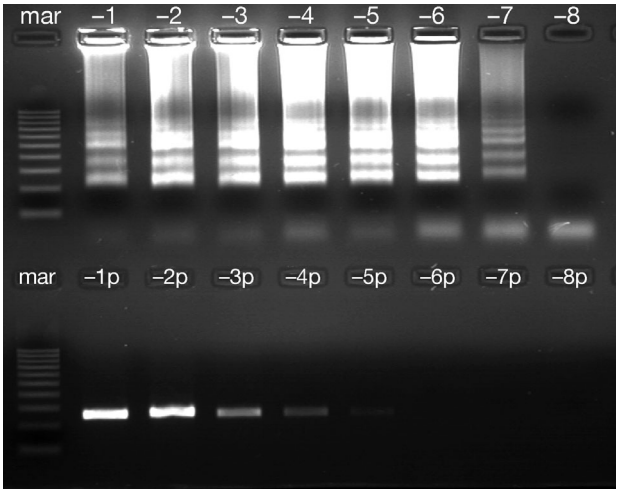

Fig. 8. *Thelohania contejeani*. Agarose gel showing lower detection limits of LAMP and PCR using 10-fold serial dilutions of template DNA. LAMP reaction detected *T. contejeani* DNA at a dilution of 10^{-7}, while PCR amplified a 261 bp product and detected *T. contejeani* DNA at a dilution of 10^{-5}. Lanes: mar = 100 bp DNA molecular weight standard; –1, –1p = template concentration 10^{-1}; –2, –2p = 10^{-2}; –3, –3p = 10^{-3}; –4, –4p = 10^{-4}; –5, –5p = 10^{-5}; –6, –6p = 10^{-6}; –7, –7p = 10^{-7}; –8, –8p = 10^{-8}

DNA in as little as 25 min (Fig. 3), we consider 60 min to be an optimal time to ensure visual detection of very low amounts of *T. contejeani* DNA; this is in accordance with the findings of Mori et al. (2001, 2004). In general, the LAMP method yielded an extremely large amount of DNA, and hence facilitated simple naked-eye inspection (Mori et al. 2001, Iwamoto et al. 2003), whereby positive samples turn green and negative samples remain orange (Fig. 4.). The sensitivity of visual detection was equivalent to that with agarose gel electrophoresis. Visual inspection has the advantages that it is simple, rapid, sensitive and requires neither post-amplification of products nor use of hazardous materials such as ethidium bromide. Thus, the LAMP assay is entirely suitable for use in a small laboratory or clinic.

The lower detection limit of the LAMP assay was 10^{-7} dilution of *Thelohania contejeani* DNA; this was superior to the PCR test which could detect down to 10^{-5} dilution. The lower sensitivity of the PCR assay (100 times less than LAMP) may be attributable to the presence of inhibitors in the samples (Ibrahim et al. 1992, Kalinina et al. 1997, Wilson 1997); the sensitivity of LAMP is not influenced by the presence of non-target DNA in samples (Notomi et al. 2000).

Our results indicate that the LAMP assay is a better alternative than PCR to traditional means for diagnosis of *Thelohania contejeani*. It detects parasite DNA with high sensitivity and requires only low-cost equipment (water bath or heating block) instead of the thermacycler and electrophoresis equipment needed for PCR. Further, as LAMP is an isothermal reaction, the reaction time is very short (60 min) and direct visual detection of LAMP reaction products is possible, requiring less labour than gel electrophoresis. We recommend the LAMP assay as a rapid diagnostic method for detection of the early stages of *T. contejeani* in asymptomatic crayfish.

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