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NOTE

Improvement of a PCR method for the detection of necrotizing hepatopancreatitis in shrimp

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ABSTRACT: Necrotizing hepatopancreatitis (NHP) is considered to be one of the most important bacterial diseases affecting penaeid shrimp culture and is caused by an unclassified Gram-negative, pleomorphic, intracellular Alphaproteobacterium. Due to the enteric nature of the bacteria, PCR is the one non-lethal method available for detection of the pathogen. Over a decade ago, a PCR protocol was developed for detection of NHP, which over the subsequent years was shown to occasionally generate false positive reactions. The University of Arizona Aquaculture Pathology Laboratory has developed a set of primers and PCR cycling parameters that have been tested on a variety of DNA templates, using 2 types of PCR reagent systems, which eliminated the generation of false positive amplicons.

KEY WORDS: PCR · Necrotizing hepatopancreatitis · NHP · Shrimp · *Penaeus vannamei*

INTRODUCTION

Necrotizing hepatopancreatitis (NHP), a disease attributed to an unclassified Gram-negative, pleomorphic, intracellular Alphaproteobacterium, affects cultured penaeid shrimp, including the Pacific white shrimp *Penaeus vannamei*. NHP causes mortalities ranging from 20 to 95% in shrimp populations not treated with antibiotics and is considered one of the most important bacterial diseases encountered in shrimp culture in the Western Hemisphere. Economic losses due to NHP have occurred in geographic areas ranging from Texas, USA, through Mexico, Central America and into South American regions where shrimp are commercially grown.

There is no method available for culturing NHP bacteria (NHPB) *in vitro*, although *in vivo* methods for experimental purposes exist. NHPB can be propagated in live shrimp by per os exposure to fresh (Vincent et al. 2004) or flash-frozen (Crabtree et al. 2006) NHPB-infected tissue. While lack of a culturing method for NHPB eliminates one possible diagnostic option, several alternate methods are available for diagnosis. Wet-mount analysis of squashed hepatopancreas (HP) can reveal melanized tubules and reduced or absent lipid droplets (Lightner 1996) that provide presumptive diagnosis, but cannot be relied on for a definitive diagnosis. Histology and *in situ* hybridization are 2 other diagnostic methods that require sacrificing the shrimp, including valuable broodstock (Lightner 1996, Loy & Frelier 1996). Fecal material contains the bacteria and allows for non-lethal sampling, but is not suitable for wet mounts, histology or *in situ* hybridization assays (Briñez et al. 2003). Thus, PCR, using fecal samples, is the preferred non-lethal method used for detection of the NHPB.

Loy et al. (1996) published a detection method for NHPB by PCR employing specific 16S rRNA primers. The method is currently used by laboratories specializing in shrimp pathogen detection in areas where NHP is endemic. The PCR method is also used in areas of Asia that recently began intensive culture of *Penaeus vannamei*. During 2006, the University of Arizona Aquaculture Pathology Laboratory (UAZAPL)

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received a request for confirmation of the presence of NHPB in penaeid species from a client from Thailand, a country that has never had a reportable case of NHP. The researchers in Thailand had used the primers and PCR protocol developed by Loy et al. (1996) for determination of NHP status, and tested extracted DNA from broodstock and juveniles from ponds. They did not state what specific reagents were used with the primers and PCR cycling protocol. Following DNA extractions at UAZAPL, the samples were tested using the primer set and PCR protocol developed for routine diagnosis at UAZAPL. NHP was not detected. The samples were tested again using the Loy et al. (1996) primers and PCR cycling protocol and extraneous bands resulted in all of the amplifications, including the specific pathogen-free (SPF) negative control, when one source of reagents was used. The present study reports on the optimized PCR primers and cycling parameters for detection of NHPB. The primers and PCR cycling protocol developed at UAZAPL were tested on various marine species and media cultured bacteria. Various variables that contribute to the false positive PCR results using the published primers developed by Loy et al. (1996) are discussed.

MATERIALS AND METHODS

DNA templates. Tissue samples of suspect NHP-infected *Penaeus vannamei* were provided by a client from Thailand. Hepatopancreata (HP), gills and pleopods from 5 pooled broodstock or juveniles were preserved and shipped in 90% ethanol. The DNA templates that were used for testing at UAZAPL were tissues from various marine species (the lobster *Panulirus argus*; *Penaeus monodon*, *P. indicus* and *P. stylirostris*; bloodworms [Polychaeta]; *Artemia* biomass; *Artemia* cysts; and red king crab *Paralithodes camtschaticus*). Additional DNA, extracted from media cultured bacteria used as templates included *Aeromonas* sp. and the *Vibrio* species *V. alginolyticus*, *V. vulniferous* and *V. parahemolyticus*.

DNA extractions. DNA was extracted from pooled hepatopancreatic tissues from Thailand as requested by the clients. DNA was also extracted from individual tissues from the various marine species and media cultured bacteria. All extractions were performed using the High Pure PCR Template Preparation Kit (Roche Diagnostics) by following the manufacturer's protocol. The DNA was eluted in a 100 µl volume and stored at –70°C until PCR amplification.

PCR amplification. Oligonucleotide primers pf1/pr1 developed from the 16S rRNA gene by Loy et al. (1996), which amplify a 441 bp fragment, and the modified PCR primers NHPF2/R2 used by the UAZAPL, which generate a 379 bp amplicon are listed in Table 1. PCR was performed in either a 50 or 25 µl volume, dependent on the reagents used in the reaction mixture. The reaction mixture (50 µl) using Applied Biosciences reagents contained: 10 mM PCR buffer (Tris-HCl, 50 mM KCl, pH 8.3), 2 mM of each deoxyribonucleotide triphosphate, 20 pmol (NHPF2/R2) or 5 pmol (pf1/pr1) of each of the 2 primers, 5.0 U AmpliTaq gold polymerase, 2 mM $MgCl_2$, 200 to 500 ng of extracted DNA and sterile H_2O. PuReTaq Ready-To-Go PCR Beads (GE Healthcare) (25 µl) were also tested and contained 30 pmol of each primer. The primers and amplification profile used followed that of Loy et al. (1996). The cycling parameters using the UAZAPL primers were as follows: 1 cycle at 95°C, followed by 25 cycles at 60°C for 30 s, 72°C for 30 s and 95°C for 30 s, and finishing with a final extension at 60°C for 1 min and 72°C for 2 min. All amplifications were performed in an Eppendorf Mastercycler. Following amplifications the PCR products were electrophoresed in 1.5% agarose gels containing 0.5 µg ml^{-1} ethidium bromide in 0.5% Tris.Borate.EDTA buffer, visualized under ultraviolet light and digitally photographed by an AlphaImager (Alpha Innotech).

Sequencing. The PCR amplicon of approximately the same size as the positive control, generated using pf1/pr1 primers and PuReTaq Ready-To-Go PCR Beads, was excised from the agarose gel. DNA was eluted from the gel slice using the QIAquick Gel Extraction Kit, following the manufacturer's protocol. The eluted DNA concentration was determined at 260 nm using a BioPhotometer (Eppendorf) and was sequenced at the University of Arizona Research Laboratory DNA Sequencing Facility.

Table 1. Primer designation, sequence, amplicon size and nucleotide location on the 16S rRNA gene

Primer designation	Primer sequence (5′ to 3′)	Amplicon size (bp)	Nucleotide in U65509
Loy et al. (1996) pf1	ACG TTG GAG GTT CGT CCT TCA G	441	752 to 773
Loy et al. (1996) pr1	TCA CCC CCT TGC TTC TCA TTG T		1171 to 1192
UAZAPL NHPF2	CGT TGG AGG TTC GTC CTT CAG T	379	753 to 774
UAZAPL NHPR2	GCC ATG AGG ACC TGA CAT CAT C		1113 to 1131

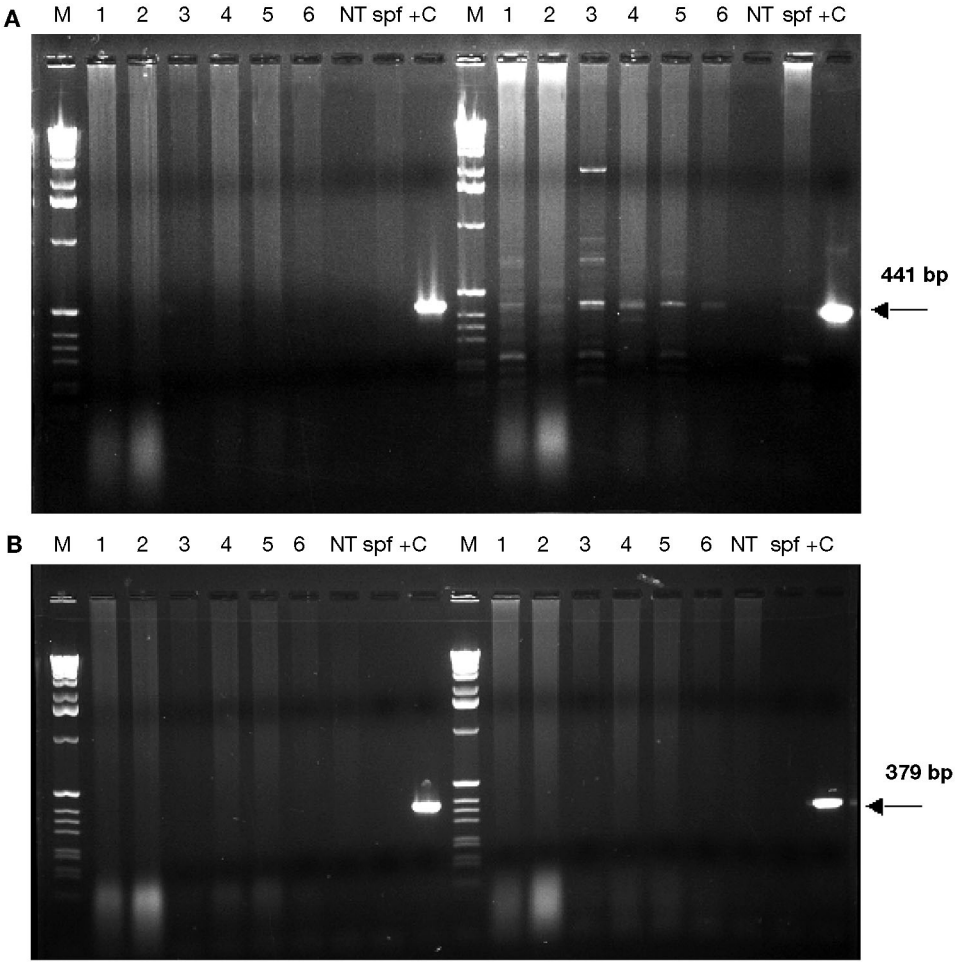

Fig. 1. *Penaeus vannamei*. Agarose gel electrophoresis analysis of PCR amplification of extracted DNA from shrimp originating from Thailand. PCR was performed using (A) Loy et al. (1996) primers and PCR cycling parameters or (B) UAZAPL primers and PCR cycling parameters. Lane M: 1 kb marker; Lanes 1 and 2: hepatopancreas; Lanes 3 and 4: gills; Lanes 5 and 6: pleopods; Lane NT: no template control; Lane spf: specific pathogen-free *P. vannamei*; Lane +C: positive control minus extracted DNA from NHP-positive *P. vannamei* sample originating from Texas. The left side of the gel shows the results of the PCR reaction when using Applied Bioscience reagents, and the right side used PuReTaq Ready-To-Go PCR Beads

RESULTS

NHP was not detected by PCR in the DNA from the extracted tissues from Thailand using either PuReTaq Ready-To-Go PCR Beads or Applied Biosciences reagents with UAZAPL diagnostic primers (Fig. 1B). This result contrasted with the result from the PCR assay using PuReTaq Ready-To-Go PCR Beads in conjunction with the primers designed by Loy et al. (1996), in which multiple bands were amplified from the extracted DNA from the HP, gills and pleopods (Fig. 1A, right side). In addition, the SPF negative control resulted in a faint fragment of approximately the same size, as would result from a positive reaction. The resultant amplified fragments, which appeared to be the same size to that of the positive control, was the reason the Thailand researchers believed the NHPB was present in the samples. When the alternative Applied Biosciences PCR reagent system and pf1/pr1 or UAZAPL primers and associated cycling parameters were tested, NHP was not detected in any of the samples except the positive control (Fig. 1).

The results from sequencing the amplicon of similar size to that of the positive control (441 bp) revealed a 395 bp sequence. This sequence was entered into GenBank, a search was performed, and no significant similarity was found with any organisms in the database. The sequence was also compared with the NHP reference sequence in GenBank (U65509). The only areas of similarity were at the 3′ and 5′ ends of the sequence.

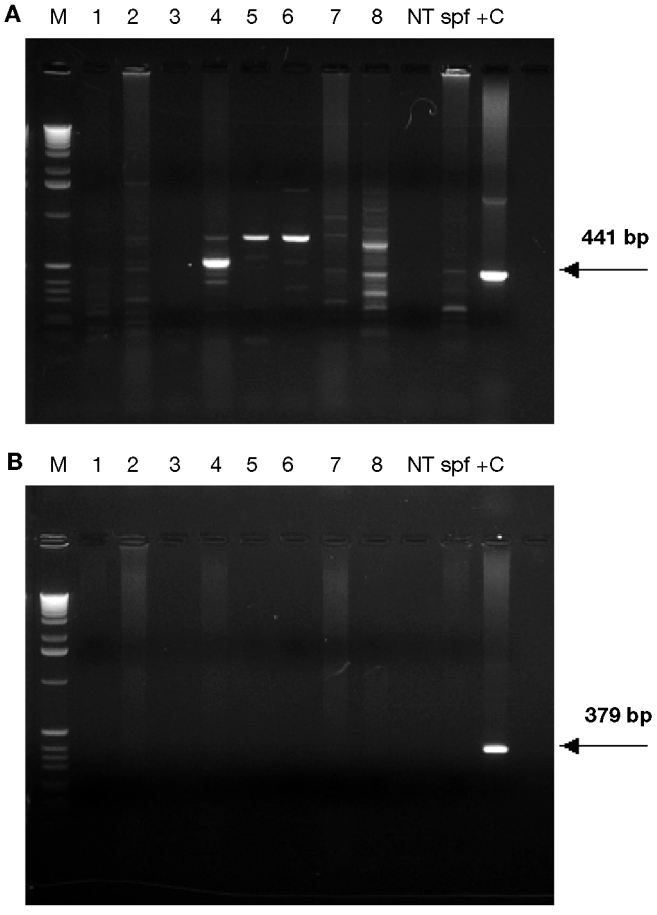

Fig. 2. Agarose gel electrophoresis analysis of PCR amplification using PuReTaq Ready-To-Go PCR Beads of extracted DNA from various marine organisms. PCR was performed using (A) Loy et al. (1996) primers and PCR cycling parameters or (B) UAZAPL primers and PCR cycling parameters. Lane M: 1 kb marker; Lane 1: lobster *Panulirus argus*; Lane 2: *Penaeus monodon*; Lane 3: bloodworms (Polychaeta); Lane 4: *Penaeus indicus*; Lane 5: *Artemia* biomass; Lane 6: *Artemia* cysts; Lane 7: red king crab *Paralithodes camtschaticus*; Lane 8: *Penaeus stylirostris*; Lane NT: no template control; Lane spf: specific pathogen free *Penaeus vannamei*; Lane +C: positive control minus extracted DNA from NHP-positive *P. vannamei* sample originating from Texas

The forward primer, pf1, had 2 mismatched bases and the reverse primer, pr1, was a 100% match (data not shown).

PCR testing of the various marine species resulted in an electrophoretic pattern consisting of multiple bands in the bloodworm, *Penaeus indicus* and *Artemia* biomass and cysts samples, using the pf1/pr1 primers with PuReTaq Ready-To-Go PCR Beads and Loy et al. (1996) cycling parameters (Fig. 2A). The multiple bands were eliminated using the same reagent system in conjunction with UAZAPL primers and PCR cycling parameters (Fig. 2B). The use of the same reagent system and Loy et al. (1996) primers and cycling parameters to amplify extracted DNA from media cultured bacteria resulted in one bright band at approximately 3000 bp, which was not seen when UAZAPL primers and cycling parameters were used. PCR amplification of the bacterial samples did not result in an amplicon of similar size to that of the NHPB positive control (data not shown).

DISCUSSION

During the development of the PCR protocol for detection of NHPB, Loy et al. (1996) tested specificity using Percoll-purified NHPB and infected *Penaeus vannamei* originating from Texas and Peru, and the results confirmed the presence of the bacteria. Additional specimens sampled and screened included *P. vannamei* infected with *Vibrio* spp. and 5 additional intracellular bacterial species. The PCR procedure was also used to test DNA extracted from healthy *P. vannamei.* The only penaeid species used to establish the specificity of the PCR assay was *P. vannamei*. The negative results from the PCR testing indicated specificity for NHPB when considering the limited panel of samples screened.

Over the course of the subsequent 10 yr, research into NHP has expanded to the investigation of vectors, hosts and environments in which the bacteria can reside. An additional complication is the culturing and sometimes co-culturing of *Penaeus vannamei* with *P. monodon* in Asia and the concern that NHP may be inadvertently introduced to this geographical area. Also, technology has continued to improve in all aspects of PCR methodology. Thermocyclers have heated lids and additional features for manipulating temperatures and cycling times. DNA extractions can be performed robotically. Sources for reagents and *Taq* polymerase have expanded. These aspects have introduced several variables that must be considered when employing a PCR test.

A primary variable introduced is the variety of sources from which DNA is extracted for use as the DNA template. While screening for vectors of NHP, false positive priming of amphipod extracted DNA was the original reason the UAZAPL developed an alternative PCR cycling protocol and primers for detection of NHP. Sequencing of the amplified product was used to determine the false positive status of the sample (University of Arizona, unpubl. data). Insects, barnacles, crabs, bloodworms, and *Artemia* also have been tested by UAZAPL using the UAZAPL NHP primers and PCR cycling protocol with PuReTaq Ready-To-Go PCR Beads. In addition, alternate penaeid species including *Penaeus stylirostris*, *P. californiensis*, *P. indicus* and *P. monodon* have been screened for NHP.

Additional variables are the plethora of methods, reagents, kits and equipment available for DNA extractions and PCR amplifications. As shown by the results from the samples from Thailand, PuReTaq Ready-To-Go PCR Beads generated multiple bands in the test samples, while use of the Applied Bioscience reagents amplified only the positive control. Multiple bands when using a single pair of primers generally indicate false priming of the DNA template. The presence of a faint band of approximately the same size as the positive control, in the SPF negative control sample, is another indication that the Loy et al. (1996) primers are amplifying a non-specific product. The use of a negative control tissue sample and no template sample are important negative controls when employing PCR as a diagnostic tool.

The PCR protocol and primers developed by Loy et al. (1996) do detect NHP nucleic acid in extracted DNA as can be seen from the positive control result. Unfortunately, this procedure has been shown to generate false positive amplicons when PuReTaq Ready-To-Go PCR Beads are used. False positive reactions using Loy et al. (1996) methodology could be a result of pf1 possessing a low stability and, thus, low selectivity. An additional cause of false positive priming could result from the relatively low annealing temperature of 52°C. The optimum annealing temperature for the primer pair pf1/pr1 is 57°C. The PCR cycling parameters and primers developed by UAZAPL are more specific than those of Loy et al. (1996) and have been tested on a variety of templates and by using 2 different reagent systems.

The results from this study reinforce the importance of a confirmatory test when unexpected results occur. PCR can be employed as a confirmatory test, but alternative primers selected from the 16S rRNA gene or from an alternative gene should be used. Standard histology, wet mounts or *in situ* hybridization assays can be confirmatory tests provided the shrimp populations have relatively high infection rates of NHPB.

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