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Detection and quantification of *Hepatobacter penaei* bacteria (NHPB) by new PCR and quantitative PCR assays

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ABSTRACT: Necrotizing hepatopancreatitis (NHP) is a bacterial disease caused by a Gram-negative bacterium classified as *Hepatobacter penaei*. *H. penaei* affects cultured penaeid shrimp in several countries from the western hemisphere, including the USA, and most Central and South American countries that farm shrimp. The current PCR and quantitative PCR (qPCR) assays based on the amplification of the 16S rRNA gene developed at the University of Arizona Aquaculture Pathology Laboratory (UAZ-APL) are the only techniques recommended in the World Organisation for Animal Health (OIE) manual for *H. penaei* detection. Although these techniques are quite sensitive and specific to *H. penaei* detection in shrimp, in recent years, rare non-specific amplifications have been observed in the end-point PCR when screening for *H. penaei* in *Artemia* cyst samples submitted to the UAZ-APL. To avoid these non-specific amplifications, new end-point PCR and qPCR assays were developed based on the *H. penaei* flagella gene, *flgE*. Unlike the current OIE methods, the new *H. penaei* PCR assay did not provide any non-specific amplification, and the qPCR assay had a detection limit of 100 copies and a log-linear range up to 10^8 copies. Because the previous PCR-based assay using the 16S rRNA was showing non-specific amplification, the new non-specific product of around 400 bp was sequenced to determine its identity. A phylogenetic analysis revealed 2 clusters of *H. penaei*: Ecuador and Central-North America. This information will enable us to determine the genetic diversity and possible origin of *H. penaei* and emphasizes the need to evaluate *H. penaei* PCR detection methods to avoid inaccurate detection of *H. penaei.*

KEY WORDS: Necrotizing hepatopancreatitis · *Penaeus vannamei* · Real-time PCR · *Hepatobacter penaei*

INTRODUCTION

Necrotizing hepatopancreatitis (NHP) is a bacterial disease of penaeid shrimp caused by a Gram-negative, intracellular pleomorphic alphaproteobacteria initially called NHPB and just recently classified as *Hepatobacter penaei* (Nunan et al. 2013), belonging to the order *Rickettsiales* in the new family Holosporales (Leyva et al. 2018). NHP affects cultured penaeid shrimp in several countries from the Americas including the USA, Mexico, Belize, El Salvador, Guatemala, Honduras, Costa Rica, Nicaragua, Panama, Brazil, Colombia, Ecuador, Peru, and Venezuela (Lightner 1996, Briñez et al. 2003, Aranguren et al. 2006, OIE 2018b). It has also been reported from an African country, Eritrea (Aranguren et al. 2010). NHP is a chronic disease that causes mortalities of up to 50–95% in affected postlarvae (PL), juveniles (Johnson 1989, Lightner 1996) and broodstock of *Penaeus vannamei* (Aranguren et al. 2006, Morales Covarrubias et al. 2006). The manifestation of NHP at farming level is related to particular environmental

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conditions such as high salinity and high temperature (Lightner 1996, Vincent & Lotz 2007). NHP-infected shrimp show a typical soft shell, flaccid bodies, reduced feed intake, and empty midguts (Lightner 1996). The acute phase lesions in diseased shrimp include necrosis and sloughing off of epithelial cells in hepatopancreas (HP) tubules, intracellular hemocytic response, and melanized HP tubules. In the chronic phase, the HP lesions are characterized by atrophy of tubules, reduced epithelial cell height, low lipid storage R cells, and intratubular edema (Lightner 1996). Since the first report in 1985 (Frelier et al. 1993), NHP has become such an important disease in the shrimp industry that in 2010 it was listed in the OIE list of crustacean diseases (OIE 2018a).

Several diagnostic methods have been developed to detect and confirm *H. penaei* presence, including PCR, histology, and *in-situ* hybridization (Lightner 1996, Loy et al. 1996, Nunan et al. 2008, Aranguren et al. 2010) and quantitative PCR (qPCR) (Vincent & Lotz 2005, Aranguren et al. 2010). However, there is only one PCR method recommended in the OIE manual (OIE 2018b), which creates the need for alternative PCR and qPCR assays for *H. penaei* detection and confirmation, especially when new non-specific amplifications are observed while screening *Artemia* cysts for *H. penaei* (see Fig. 1).

Bacterial flagella are complex and well-honed organelles that provide swimming and swarming motilities and play a central role in adhesion, biofilm formation, and host invasion (Kirov 2003). The typical bacterial flagellum consists of 6 components: a basal body (including MS ring, P ring, and L ring), a motor, a switch, a hook, a filament, and an export apparatus (Macnab 2003). The core set of flagellar genes, which is uniformly present in all flagellated bacteria, has evolved and diverged in a lineage-specific manner (Liu & Ochman 2007). This makes flagellar genes highly specific for a specific bacterial group. Here we describe PCR-based detection methods targeting flagellar genes that are specific for *H. penaei* and do not provide non-specific amplification in *Artemia* samples.

The purpose of the present study was to develop new PCR and qPCR methods for *H. penaei* detection with high sensitivity and higher specificity in comparison with the current OIE method (OIE 2018b). In addition, the cause of non-specific amplification while screening samples for *H. penaei* based on 16S rRNA gene was investigated. Lastly, we investigated possible clades among *H. penaei* isolates through a phylogenetic analysis. Considering the diversity that exists among geographical isolates of *H. penaei* and the need to develop an alternative method to avoid non-specific amplification, the detection methods described here are major improvements in *H. penaei* screening.

MATERIALS AND METHODS

The *Hepatobacter penaei*-positive samples used in this study originated in Texas, USA (samples a & b from 2006), Ecuador (2011), Sonora, Mexico (2013), Texas, USA (samples a, b & c from 2013), Ecuador (samples a & b from 2015), and Honduras (2016). These samples are archived at the University of Arizona Aquaculture Pathology laboratory (UAZ-APL).

HP samples were preserved in 95% ethanol. *Artemia* samples were shipped to UAZ-APL by the clients either in a commercially available container (454 g size can) or as aliquots of the cysts from a can in plastic bags (~50 to 100 g size). From each can or plastic bag, 3 sub-samples were collected from the top, middle and bottom of the container/bag. The three sub-samples were then mixed and 25 to 50 mg was taken for the isolation of DNA.

DNA extraction

DNA extraction was carried out from HP and *Artemia* cysts. Approximately 25 to 50 mg of HP tissue/*Artemia* cysts was taken for DNA extraction using a Maxwell® 16 Cell LEV DNA Purification Kit following the manufacturer's protocol. Upon extraction, the DNA was stored at −20°C until further analysis.

PCR

The nucleotide sequence of the flagellar hook protein gene *flgE* of *H. penaei* (GenBank accession number: JQAJ01000001.1) that encodes for the hook subunit of the flagellum was used to design *H. penaei* primers using Primer Express 3.0 (Applied Biosystems). PuReTaq Ready-To-Go PCR beads were used for the PCR assay. The primers for detecting the *H. penaei* were NHP FlgE 1143F (5′-AGG CAA ACA AAC AAC CCT TG-3′) and the NHP FlgE 1475R (5′-GCG TTG TTG TTG GGA AAG TT-3′). Amplifications were performed with the following cycling parameters: initial denaturation at 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 s, 62ºC for 30 s, and 72ºC for 30 s, and a final extension at 72ºC for 5 min

to generate an amplicon of 333 bp from the NHP *flgE* gene region.

To test the sensitivity of the NHP FlgE primers, PCR assays were performed using DNA from 10 NHP-positive samples following the new assay vs. the conventional PCR protocol recommended by OIE (Aranguren et al. 2010).

To test the specificity of the NHP FlgE primers, PCR assays were performed with DNA isolated from shrimp infected with 5 different bacterial pathogens: *H. penaei*, *Vibrio parahaemolyticus*, *V. harveyi*, *Spiroplasma penaei*, and *V. parahaemolyticus*, which causes acute hepatopancreatic necrosis disease / early mortality syndrome (AHPND/EMS). In addition, DNA samples isolated from shrimp infected with the viruses infectious myonecrosis virus (IMNV), yellow head virus (YHV) genotype 1, infectious hypodermal hematopoietic necrosis virus (IHHNV), white spot syndrome virus (WSSV), and Taura syndrome virus (TSV) were tested. DNA isolated from specific pathogen free (SPF) *Penaeus vannamei* was taken as a negative control for the PCR assay. In addition, samples of *Artemia* cysts that displayed non-specific amplification using the OIE method were run with the NHP FlgE primers. The unusual bands were purified from the electrophoresis gel using the QIAquick PCR purification kit (Qiagen) and sent for sequencing in both directions.

qPCR

The primers and the TaqMan hydrolysis probe were designed using the Primer Express software version 3.0 (Applied Biosystems) from the flagella hook gene *flgE* of *H. penaei*. Primers (NHP FlgE3qF: 5′-AAC ACC CTG TCT CCC CAA TTC-3′; and NHP FlgE3qR: 5′-CCA GCC TTG GAC AAA CAC CTT-3′) were used to produce a PCR product of 63 bp. The TaqMan probe, NHP: 5′-CGC CCC AAA GCA TGC CGC-3′, was synthesized and labeled with the fluorescent dyes 6-carboxyfluorescein (FAM) on the 5′ and N,N,N,N-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′ end. The amplification reactions were conducted as follow: 0.5 µM of each primer, 0.1 µM of TaqMan probe, 1× TaqMan Fast Virus 1-Step Master Mix (Life Technologies), 5 to 50 ng of DNA and HPLC water in a reaction volume of 10 µl. The qPCR profile consisted of 20 s at 95ºC followed by 40 cycles of 1 s at 95ºC and 20 s at 60ºC. Amplification detection and data analysis for qPCR assays were carried out with the StepOnePlus real-time PCR system (Life Technologies).

Generating a standard curve for quantification of *H. penaei*

To generate a plasmid DNA standard curve for the NHP *flgE* qPCR assay, the primers NHP FlgE 1143F and 1475R were used to amplify a 333 bp product from the NHP *flgE* gene. The PCR product was cleaned using the QIAquick PCR Purification Kit (Qiagen). The amplicon was cloned into a pCR2.1-TOPO TA vector (Invitrogen) and transformed into *Escherichia coli* JM109 cells (Promega). The recombinant plasmid pNHPFlgE was verified by DNA sequencing in both orientations with an automated Applied Biosystems 3730 DNA Analyzer. The concentration of pNHPFlgE was determined by measuring the optical density (OD) at 260 nm in a Nanodrop™ spectrophotometer. Each sample was measured 5 times and the mean OD was used to estimate the amount of DNA. The DNA quality was checked using the 260:280 ratio. The DNA copy number was calculated according to Staroscik (2004): (amount of DNA in ng) × (Avogadro's number) / (650 Da) × (length of template in bp). In order to determine the analytical sensitivity of the qPCR, serial dilutions of purified plasmid pNHPFlgE were conducted. The plasmid DNA was diluted 10-fold from 10^8 copies to 1 copy per reaction in 6 independent assays to determine reproducibility. All the dilutions were conducted using DNA from a SPF shrimp *P. vannamei* at a concentration of 10 ng μl^{-1} as a carrier (Aranguren et al. 2010)

Phylogenetic analysis

Oligonucleotide primers NHP16S rRNA F2 (5′-GTG GCA GAC GGG TGA GTA AT-3′) and NHP16S rRNA R2 (5′-CCT CCA TTG CTG GTT AGC TC-3′), which generate a 1321 bp amplicon, were used to compare the different *H. penaei* isolates by PCR. The primer concentration for both (F2/R2) was 0.2 µM. The PCR was carried out using PuReTaq Ready-To-Go PCR beads (GE Healthcare) in a final volume of 25 µl. Amplifications were performed with the following parameters: initiation denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 5 min. An aliquot of PCR products was analyzed in a 1.5% gel containing 0.5 µg ml^{-1} ethidium bromide.

The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen) and sent for sequencing in both directions. Assembly of the sequence

was carried out by overlapping amplified regions using Geneious 4.8.5 software, with NHP 16S rRNA (GenBank accession number: U65509.1) (Loy et al. 1996) as a template. Multiple sequence alignment and phylogenetic analyses were performed with Clustal X (Thompson et al. 1997). The phylogenetic tree was inferred by using the maximum likelihood method based on the Tamura-Nei model (Tamura & Nei 1993). The bootstrap consensus tree was inferred from 1000 replicates (Kuhner et al. 1995). The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1131 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). Evolutionary divergence between sequences was determined using the maximum composite likelihood model.

RESULTS

Non-specific amplification

The PCR analysis of 2 samples of *Artemia* cysts with the 16S rRNA primers resulted in non-specific bands of about 400 bp (Fig. 1, left side). In contrast, when the same sample set was amplified with the FlgE primers, no amplification products were visible in the electrophoresis gel (Fig. 1, right side). The sequencing data revealed that the amplicon obtained using the 16S rRNA primers did not correspond to *Hepatobacter penaei*, confirming non-specific amplification in samples of *Artemia* cysts. The nature of the non-specific product from *Artemia* samples was further analyzed by comparing the amplicon sequence with the NCBI database using BLASTN analysis. The sequence from the sample coded 17-689 showed highest alignment with the bacterium *Halanaerobium* sp. (98% identity) (GenBank accession number: FJ858788.1). The best alignment of the sequence from sample 18-316 was with an uncultured bacterium (96% identity) (JX882300.1).

Analytical sensitivity and specificity of qPCR

The analytical sensitivity of the NHP PCR was determined by using *H. penaei* in 10 different independent samples from 7 origins with 2 different sets of primers: 16S rRNA and FlgE (Fig. 2). The left side of the gel in Fig. 2 shows samples amplified using the current OIE-recommended method (16S rRNA); the right side shows the same samples amplified by the new method. Both methods provided similar results, and the gel bands had similar intensity. Sample 12 (Brazil 2012) showed very weak amplification in both protocols, which suggests partial DNA degradation. Despite this, the conventional PCR method based on the *flgE* gene is comparable to the 16S rRNA-based PCR.

In order to determine the specificity of amplification using the *flgE* protocol, DNA samples extracted from shrimp infected with viruses, including IMNV, YHV, IHHNV, WSSV, and TSV, and bacteria, including *Vibrio parahaemolyticus*, *V. harveyi*, *Spiroplasma penaei*, *V. parahaemolyticus* (causing AHPND), and *H. penaei*, were used as templates for PCR amplification. Negative results were obtained for all shrimp diseases except *H. penaei*. (Fig. 3). This indicates the specificity of the primers for the *flgE* gene, although only a narrow range of Gram-negative bacteria are known to affect shrimp.

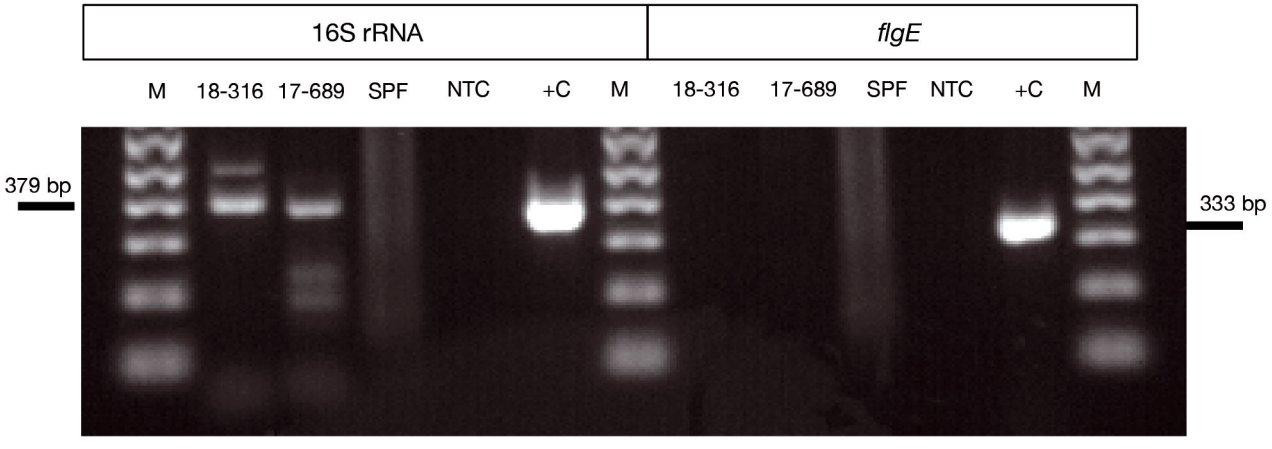

Fig. 1. PCR detection of *Hepatobacter penaei* in samples of *Artemia* cysts targeting 2 different genes: 16S rRNA and *flgE*. M: 1 kb plus ladder molecular weight marker; 18-316: case 1; 17-689: case 2; NTC: no template control; +C: *H. penaei* positive control

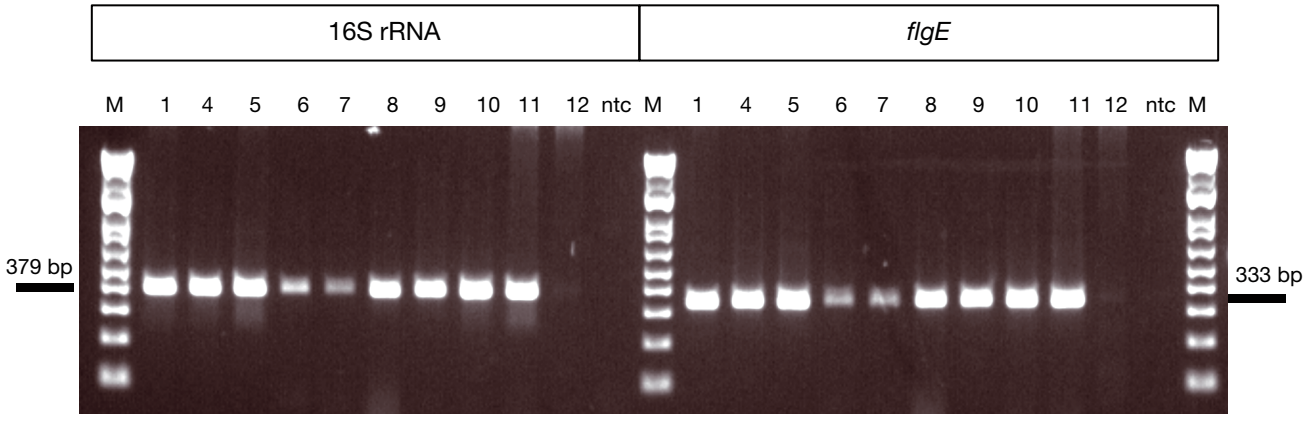

Fig. 2. PCR detection of *Hepatobacter penaei* in 10 different samples of infected *Penaeus vannamei* hepatopancreas based on 16S rRNA and *flgE* genes. M: 1 kb plus ladder molecular weight marker; ntc: no template control; 1: Ecuador 2015a; 4: Mexico 2013; 5: Texas 2013a; 6: Belize 2016; 7: Panama 2016; 8: Texas 2006a; 9: Honduras 2016; 10: Texas 2006b; 11: Texas 2013b; 12: Brazil 2012

Analytical sensitivity of qPCR

The analytical sensitivity of the qPCR protocol for *H. penaei* detection was determined using a plasmid containing a 333 bp fragment of the *flgE* gene including the target sequence. A 10-fold dilution series of the plasmid, corresponding to 1×10^0 to 1×10^8 copies, was tested. *H. penaei* was detected at 100 copies in all 6 assays (Table 1). Therefore, the detection limit was considered to be 100 copies (Fig. 4). The reproducibility of this new qPCR protocol was confirmed by 6 independent standard curves compared in a 7 log range from 1×10^2 to 1×10^8 copies per reaction (Table 1).

The standard deviation (SD) within each run ranged from 0.0 to 0.7, with the highest SD in 10^3 copies. The combined data from the 6 independent assays did not show a linear relationship between the quantification cycle (Cq) values and the SD ($p > 0.05$). The SD values for the inter-assay replicates ranged between 0.3 and 0.9, indicating high reliability of this assay (Table 1). The correlation coefficient (R^2) of the standard curves was greater than 0.99 in all the cases, also indicating good reproducibility. The reaction efficiency values ranged from 0.99 through to 1.08 with a mean slope of −3.23 and an intercept of 40.58, indicating high efficiency of the PCR and, hence, good optimization of the protocol (Fig. 4).

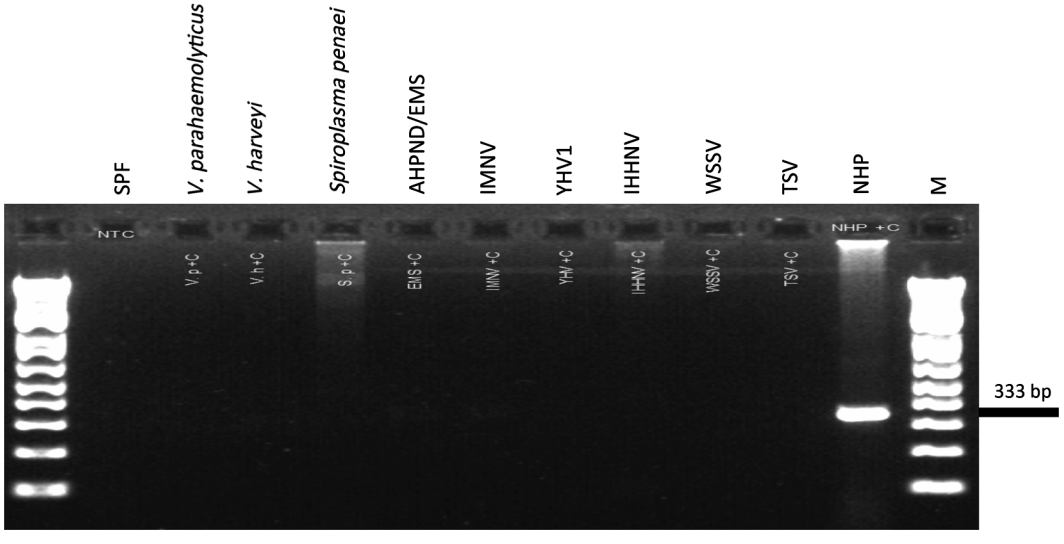

Fig. 3. PCR detection of *Hepatobacter penaei* in positive controls of shrimp *Penaeus vannamei* infected with pathogens including *Vibrio parahaemolyticus, V. harveyi, Spiroplasma penaei*, *V. parahaemolyticus* (AHPND/EMS), IMNV, YHV genotype 1, IHHNV, WSSV, TSV, and *H. penaei* (NHP). A specific pathogen free (SPF) sample was included in the assay as a negative control. M: 1 kb plus ladder molecular weight marker

Table 1. Reproducibility of the TaqMan qPCR assay for detecting *Hepatobacter penaei* (NHP) in 6 different assays. SD: standard deviation; Cq: quantification cycle

NHP copies	Intra-assay SD (average Cq value for duplicate)												Inter-assay SD (mean Cq value)	
	1		2		3		4		5		6			
1×10^2	0.5	(34.6)	0.1	(34.9)	0.0	(32.9)	0.1	(34.1)	0.5	(33.6)	0.0	(35.4)	0.90	(34.2)
1×10^3	0.1	(31.1)	0.2	(31.3)	0.3	(30.0)	0.7	(30.2)	0.3	(30.9)	0.1	(31.0)	0.54	(30.8)
1×10^4	0.1	(28.0)	0.2	(28.0)	0.1	(27.5)	0.3	(26.5)	0.5	(27.5)	0.2	(27.8)	0.57	(27.5)
1×10^5	0.0	(24.7)	0.2	(25.1)	0.0	(24.4)	0.5	(23.4)	0.2	(25.1)	0.1	(24.4)	0.63	(24.5)
1×10^6	0.1	(21.2)	0.0	(22.0)	0.0	(21.1)	0.3	(20.8)	0.2	(21.1)	0.2	(21.1)	0.43	(21.2)
1×10^7	0.0	(18.0)	0.5	(18.5)	0.2	(17.8)	0.2	(17.8)	0.4	(17.6)	0.1	(17.9)	0.30	(17.9)
1×10^8	0.0	(14.9)	0.2	(15.2)	0.0	(14.4)	0.0	(14.4)	0.5	(14.5)	0.4	(14.6)	0.32	(14.7)

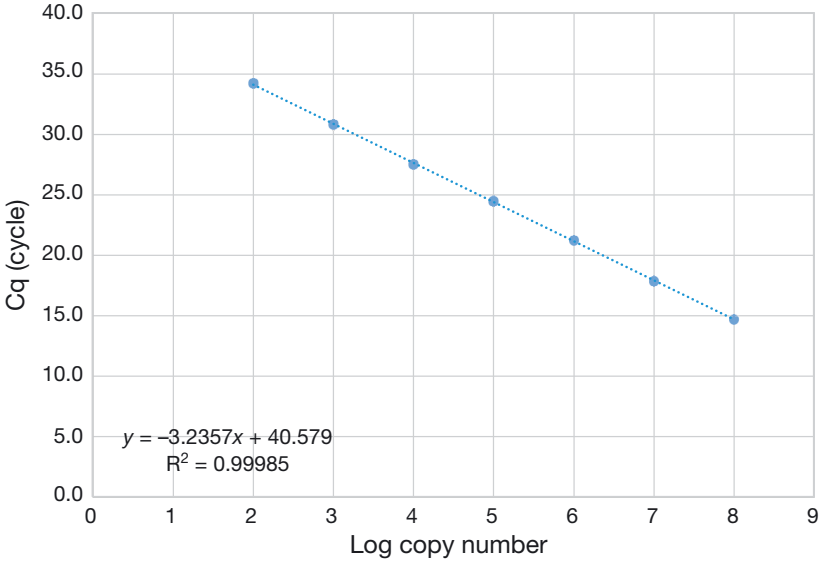

Fig. 4. Standard curve of the *Hepatobacter penaei* copy number versus quantification cycle (Cq) value. Purified pNHPFlgE plasmid was serially diluted from 1×10^8 to 100 copies and used as a template in qPCR

Comparison of NHP 16S rRNA and *flgE* qPCR

To determine the difference in sensitivity between the new NHP FlgE assays and the current OIE-recommended end-point PCR method, a strong positive sample with a high copy number (2.06×10^6 *H. penaei* copies μl^{-1} DNA) was used in serial dilutions from 10^6 through to 10^1. This series of samples was amplified by both PCR and qPCR. The end-point PCR could detect down to 2.06×10^3 copies reliably, and the sample containing 2.06×10^2 copies provided a faint band (a weak positive result) (Fig. 5). Samples with a lower bacterial density (2.06×10^1 copies μl^{-1} DNA and less) were not detected with the

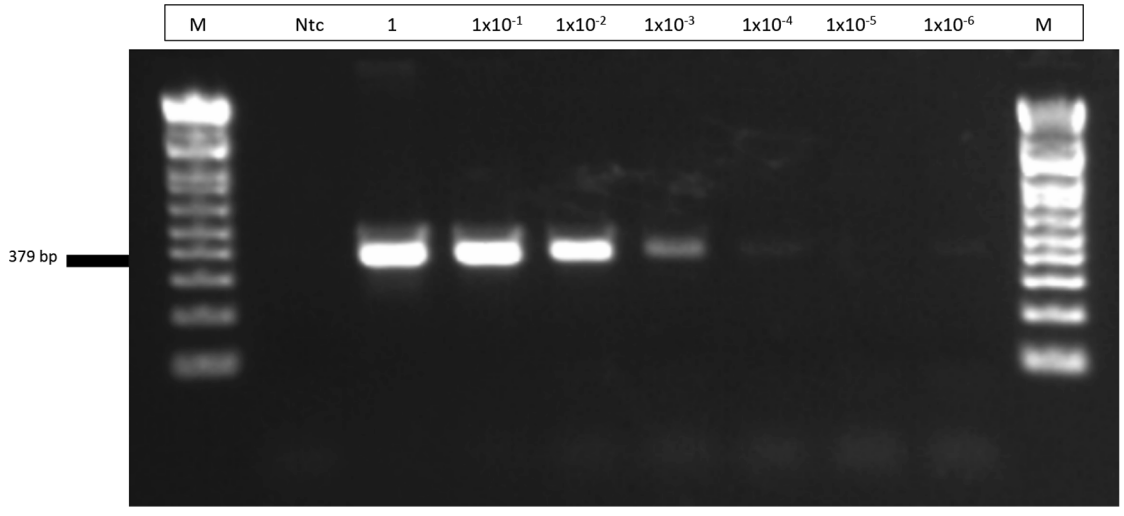

Fig. 5. Amplification of *Hepatobacter penaei* using conventional PCR based on the NHP 16S rRNA gene and using 6 serially diluted samples of template DNA. Sample 1 contained 2.06×10^6 NHP copies μl^{-1} DNA. Ntc: no template control; M: 100 bp ladder

H. penaei PCR assay. When qPCR was used, samples diluted up to 1 × 10^{-5} (2.06 × 10^{1} copies μl^{-1} DNA) could be detected (Fig. 6).

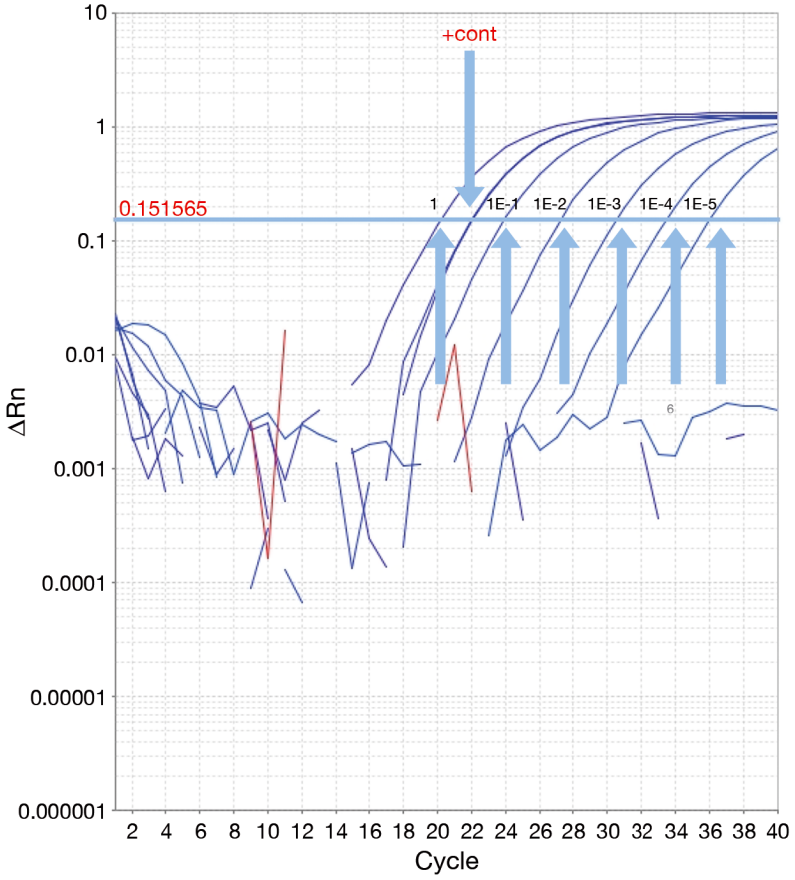

Fig. 6. Threshold cycle number (Ct) curves for *Hepatobacter penaei* amplification using qPCR based on the NHP *flgE* gene and using 6 serially diluted template DNA. Sample 1 contained 2.06 × 10^{6} NHP copies μl^{-1} DNA. Rn: normalized reported value

Phylogenetic analysis of the NHP 16S rRNA sequence

A phylogenetic tree was constructed based on the nucleotide sequences of the 1.131 kb amplicon of 16S rRNA obtained for 12 *H. penaei* isolates (Fig. 7). The NHP sequences clustered into 2 clear groups. In one clade, samples from Ecuador, EC 2011 (GenBank accession number: MH230903), EC 2015a (MH230902), and EC 2015b (MH230909), grouped together with a bootstrap value of 64%. In the other, samples from Mexico, SON-MX 2013 (MH230904), Honduras, HN 2016 (MH230907) and the Texas, USA, TX-US 2006a (MH 230906), TX-US 2006b (MH230900), TX-US 2013a (MH230908), and TX-US 2013c (MH230905), clustered together with a bootstrap value of 64% as well. One sample from Mexico, MX 2013 (JX981946), did not group with the other MX isolate. One sample from Texas, TX-US 2013b (MH 230901), did not group with the other Texas samples. An NHP sequence from 1996 (U65509.1) that was used as the root sequence differed from the new NHP sequences from 2006 to 2016 (Fig. 7). The number of base substitutions per site between sequences are shown in Table 2. The nucleotide identity among the NHP 16S rRNA sequences ranged between 99.64 and 100.00%.

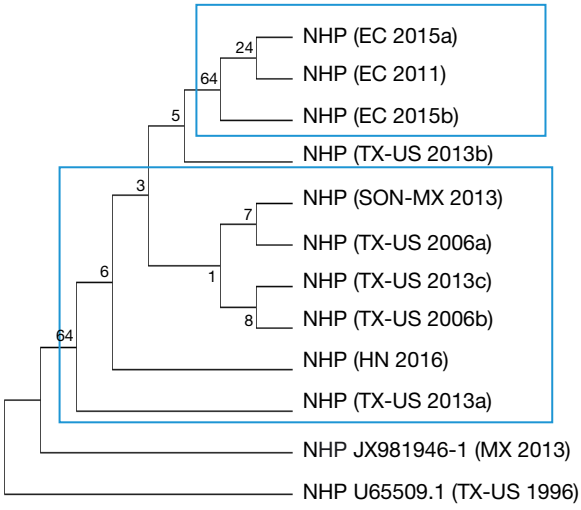

Fig. 7. Maximum likelihood phylogenetic tree based on the Tamura-Nei model from the alignment of the 16S rRNA sequences among 12 *Hepatobacter penaei* (NHP) geographical isolates (abbreviation and year; EC: Ecuador; TX-US: Texas, USA; SON-MX: Mexico; HN: Honduras). Blue squares highlight the 2 clusters. Bootstrap values (>50%), generated from 1000 replicates, are indicated next to the branches

DISCUSSION

In order to minimize the introduction of shrimp pathogens into shrimp-producing countries, a series of biosecurity strategies have been developed, including testing shrimp and shrimp-related products for the OIE-listed pathogens that affect shrimp. The variety of samples have been challenging for the PCR diagnosis laboratory in terms of the presence of unexpected results, including the recently observed non-specific bands for *Artemia* cysts reported here.

Table 2. Estimates of nucleotide similarity among geographical isolates of *Hepatobacter penaei* based on the 16S region

Sequence ID	EC 2015a	EC 2011	SON-MX 2013	TX-US 2013c	TX-US 2006a	HN 2016	TX-US 1996	TX-US 2013b	TX-US 2006b	EC 2015b	TX-US 2013a	MX 2013
EC 2015a	–											
EC 2011	100.00	–										
SON-MX 2013	99.91	99.91	–									
TX-US 2013c	99.91	99.91	100.00	–								
TX-US 2006a	99.91	99.91	100.00	100.00	–							
HN 2016	99.82	99.82	99.91	99.91	99.91	–						
TX-US 1996	99.82	99.82	99.91	99.91	99.91	99.82	–					
TX-US 2013b	99.91	99.91	100.00	100.00	100.00	99.91	99.91	–				
TX-US 2006b	99.91	99.91	100.00	100.00	100.00	99.91	99.91	100.00	–			
EC 2015b	100.00	100.00	99.91	99.91	99.91	99.82	99.82	99.91	99.91	–		
TX-US 2013a	99.91	99.91	100.00	100.00	100.00	99.91	99.91	100.00	100.00	99.91	–	
MX 2013	99.64	99.64	99.73	99.73	99.73	99.64	99.82	99.73	99.73	99.64	99.73	–

New PCR and qPCR assays were developed to detect and quantify *Hepatobacter penaei* in shrimp tissues using the NHP *flgE* gene. This new end-point PCR assay has a similar sensitivity to the currently recommended OIE *H. penaei* assay (OIE 2018b). However, the specificity of the new PCR assay is higher, and the non-specific positive results obtained when targeting the 16S rRNA gene were not seen. This could be explained by the fact that the target gene for the PCR, *flgE*, shows a greater specificity compared to the 16S rRNA gene. As a result, PCR based on the *flgE g*ene is unlikely to provide false positive results in the variety of samples, including *Artemia* cysts and shrimp tissues, analyzed by the UAZ-APL.

The non-specific amplification of *Artemia* samples using the OIE *H. penaei* assay is due to the possible presence of uncultured bacteria living in hypersaline conditions with a 16S rRNA sequence similar to *H. penaei*. *Artemia* cysts are naturally present in hypersaline environments (FAO 2011); hence, it is likely that some bacteria remain attached to or inside the cysts during the harvesting process. Considering the fact that field-collected shrimp samples and shrimp-related samples often carry more than one pathogen, the specificity of the newly developed methods will enable us to avoid non-specific amplifications. In addition, when evaluating the specificity using other shrimp pathogens, the only positive result was found with the *H. penaei* isolate, indicating the high specificity of this assay.

The new qPCR developed in this study had a detection limit of 1×10^2 copies. A comparison between the OIE qPCR method versus this new qPCR assay showed consistent results and similar Cq values (data not show). In addition, samples from 4 different geographical areas, including Mexico, the USA, Honduras, and Ecuador, collected in different years confirmed the specificity of this new qPCR. So far, only 2 previous studies have reported *H. penaei* quantification in shrimp tissue (Vincent & Lotz 2005, Aranguren et al. 2010). While attempting to reproduce the results reported by Vincent & Lotz (2005), inconsistent values for the *H. penaei* copy number were obtained (Aranguren et al. 2010). Later, a more reproducible method was developed by the UAZ-APL (Aranguren et al. 2010). However, as the target region is based on the 16S rRNA gene, some non-specific amplification may be expected. The qPCR method based on the *flgE* gene will enable us to avoid this scenario.

The phylogenetic analysis based on the 16S rRNA gene showed 2 clusters, one containing isolates from Ecuador, and the other containing isolates from Mexico, Honduras, and Texas, USA. The clustering of the samples from Ecuador taken in 2011 and 2015 suggests the presence of the same *H. penaei* isolate. Another possibility is a low mutation rate of this *H. penaei*. The observation that some samples from Texas, USA (2006 & 2013) and Sonora, Mexico (2013) grouped together indicates that the same *H. penaei* isolate could be present in both countries due to the marine currents and/or movement of NHP-infected shrimp between the 2 geographically separated areas. The ability to detect *H. penaei* in a wide range of geographical isolates will be useful for routine screening of *H. penaei*, and to avoid false negatives.

NHP has been reported in sub-adults and broodstock populations in farms (Aranguren et al. 2006) and in juveniles (Lightner 1996), causing different mortality patterns (Briñez et al. 2003, Aranguren et al. 2010). In some countries, such as in Colombia, NHP does not cause the acute mortalities reported in other countries, and the typical NHP clinical signs

are not always detected (Aranguren et al. 2006). This phenomenon could be related to the resistance of some *Penaeus vannamei* lines to NHP (Aranguren et al. 2010). It is also possible that *H. penaei* isolates may vary in their pathogenicity, which could explain the different mortality patterns in different regions. The ability to detect down to 100 copies in the qPCR assay based on the *flgE* gene will be very useful for detecting *H. penaei* in samples that display subclinical signs or no signs at all.

In summary, we describe conventional PCR and qPCR assays as alternative methods for the diagnosis and quantification of *H. penaei* in shrimp and shrimp-associated samples. The assays are highly specific and sensitive. The sensitivity of the NHP FlgE PCR is similar to the currently recommended OIE method, but it is more specific and sensitive; hence it may be a better diagnostic tool for this pathogen.

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