The following supplement accompanies the article

Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico

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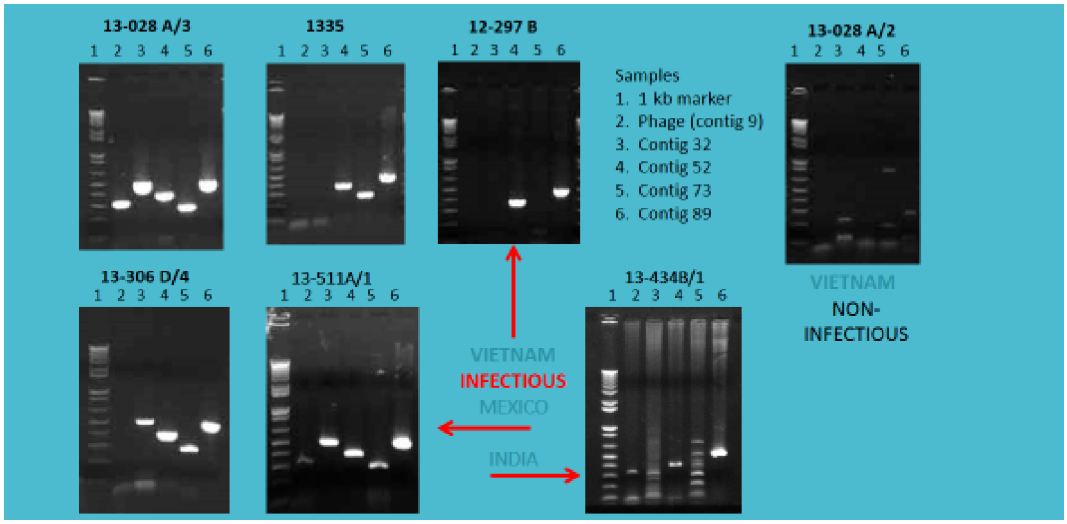
Diseases of Aquatic Organisms Series 111: 81–86 (2014)

Supplement.

This attachment provides supplemental information on the tests performed for sensitivity and specificity of the PCR assay designed for detection of the pathogenic agent responsible for acute hepatopancreatic necrosis desease (AHPND). After Lightner's laboratory determined that the bacterium causing AHPND was *Vibrio parahaemolyticus*, the corresponding author realized that in order to target the specific isolate(s) that cause the disease, the PCR test would have to be based on some element that had either been incorporated into the genome or added to genomic material by way of mobile genetic elements. The first step in obtaining this information was to sequence the AHPND-causing bacterium *V. parahaemolyticus*, 13-028A/3 (A/3), which we were the first to isolate and which was described in Tran's (2013) paper. At the same time, 13-028A/2 (A/2), a non-pathogentic *V. parahaemolyticus*, was metagenomically sequenced.

When the sequence data were returned, contigs were examined for unique sequences not found in the complete genomes of the reference *V. parahaemolyticus* material from the GenBank database. Five large contigs did not relate directly to the reference *V. parahaemolyticus*, and PCR primers were designed to the specific contigs. Contig 9 was a phage, Contig 32 appeared to be a prophage, and Contigs 52, 73 and 89 all appeared to be plasmids. By this point in time, we had already isolated several additional AHPND-causing isolates, so these were included in the PCR testing with specific primers (Fig. S1).

Fig. S1. PCR assays determining the presence of various contigs in several AHPND-causing *V. parahaemolyticus* samples



Isolates 13-028A/3 (also referred to as 'A/3'), 1335 and 12-297B originated from Asia. Isolates 13-306D/4 and 13-511A/1 originated from Mexico. These 5 bacterial isolates were confirmed by histology, challenge studies and PCR. The template for PCR from these 5 isolates was broth culture, used directly, with no DNA extractions performed. Isolate 13-434B/1 originated from India. This was a tissue sample from which DNA was extracted from ethanol-preserved HO. Isolate 13-028A/2 (also referred to as 'A/2') was the non-pathogenic *V. parahaemolyticus.*

The results from these PCR assays showed that, as expected, isolate A/3 contained all the 'unique' contigs, while the non-pathogenic *V. parahaemolyticus* contained none of the extra genetic material. The results also helped narrow down the sequence that would be targeted for a specific PCR assay. Table S1 summarizes the results from these experiments.

Table S1. Bacteria or extracted DNA tested for the presence of various unique contigs by PCR

Isolate	Contigs				
	9	32	52	73	89
A/3	+	+	+	+	+
1335	-	-	+	+	+
B	-	-	+	-	+
D/4	-	+	+	+	+
A/1	+	+	+	+	+
13-434B/1	+	-	+	-	+
A/2	-	-	-	-	-

These results indicated that only Contigs 52 and 89 were present in all of the AHPND-causing *V. parahaemolyticus*.

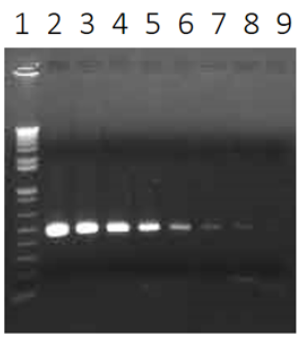
Contig 89 was the specific target chosen due to a small experiment performed in which the corresponding author attempted to transfer 52 and 89 sequences (assuming the plasmid was transferred also) into the non-pathogenic A/2 isolate. Contig 52 transferred, but Contig 89 did not. The bacteria with 52 transferred was tested by challenge and did not cause AHPND, so 89 was targeted.

To determine the sensitivity of the PCR assay, an A/3 TSB+ broth culture that had been incubated overnight at 28°C was used. The following day, the culture was diluted to 10^{-9} in tryptic soy broth plus 2% NaCl (TSB+). Two dilutions (100 ml), in triplicate, were plated on TSA+, at 10^{-5} and 10^{-6} dilutions. The dilutions were also used as templates for the PCR reactions. No extractions were performed, and 1.0 ml was added to each reaction tube that contained a Ready-To-Go PCR bead with 1.0 ml (0.25 mM each) of the primer mix (89F/R) added. Refer to the manuscript for PCR running parameters. Fig. S2 summarizes the results from this PCR assay and plate counts.

Fig. S2. AHPND bacterial dilutions tested by PCR for the presence of AHPND using primers 89F/R and bacteria, directly, from each dilution. Dilutions 10^{-5} and 10^{-6} were also plated, and the count-forming units (CFU) for the dilutions were determined.

Samples

1. 1 kb marker
2. A/3 (10^{-2})
3. A/3 (10^{-3})
4. A/3 (10^{-4})
5. A/3 (10^{-5})
6. A/3 (10^{-6})
7. A/3 (10^{-7})
8. A/3 (10^{-8})
9. A/3 (10^{-9})

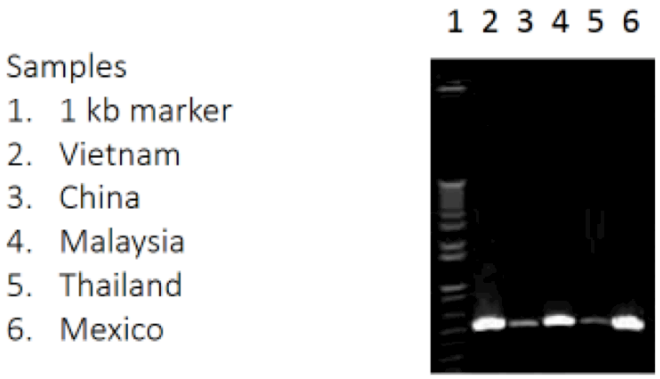


13-028 A/3 dilution	Plate count results			PCR copy #
	Replicate	# colony forming units (CFU)	Mean CFU/ml	1 µl template
10^{-5}	#1	249	2460	2.46
	#2	234		
	#3	256		
10^{-6}	#1	19	460	0.46
	#2	89		
	#3	29		

By gel analysis the 10^{-6} dilution would be considered positive, albeit weak. This dilution was equal to 460 CFU, when 100 ml was plated on TSA+. One microliter of that same dilution was used for the PCR templates, so PCR detected 4.6 bacteria in the sample. Sensitivity for the assay using broth culture is in the 1 to 10 bacteria range.

The PCR assay appeared to be sensitive, at least when using broth culture as the template, but the detection method also needed to be tested using DNA extracted preferably from the HPs, but, if necessary, from the stomachs. In addition, it was necessary to know if the assay could detect AHPND-causing isolates, in frozen or ethanol-preserved samples, from various geographical locations. Initially, frozen material that UAZ-APL had received over the previous months from AHPND endemic areas in Southeast Asia was tested. The HPs from the frozen *P. vannamei* were excised, usually in pools of 5. DNA was extracted using the High Pure PCR Template Preparation Kit (Roche). PCR was run as previously described using 1.0 ml of the extracted DNA (Fig. S3).

Fig. S3. PCR gel electrophoresis results using primers 89F/R for detection of AHPND in the HPs of frozen tissue originating from various geographical locations



LITERATURE CITED

Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, Lightner DV (2013) Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Org 105:45–55