Novel Diagnostic Tests for the Putative Agent of Bacterial Gill Disease in Pacific Razor Clams (*Siliqua patula*)

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Abstract

Nuclear inclusion X (NIX) is a gamma proteobacteria that infects the nuclei of gill epithelial cells in Pacific razor clams. NIX has been associated with clam die-offs in coastal Washington. A quantitative PCR (qPCR) assay was developed to detect NIX in Pacific razor clams, and assay specificity was confirmed by chromogenic in situ hybridization (CISH). Both tests were applied to evaluate NIX infections in wild Pacific razor clams collected during spring 2019. Consistent with results from earlier histopathological assessments, qPCR and CISH indicated 100% prevalence in razor clams from two Washington beaches and 0% prevalence from two Alaskan beaches.

Keywords: Razor Clam, Nuclear Inclusion X, NIX

1. Introduction

Pacific razor clam (*Siliqua patula*) mass mortality events occurred along coastal beaches of Washington (USA) during the 1980s and early 2000s. Associated population declines exceeded 95% in a survey from 1983-1984 (Elston et al. 1986). From 2006-2008, low survival of older clams, and depressed population size resulted in the decision to close the recreational razor clam fishery at Kalaloch beach (Fradkin et al. 2018). Although the causes of these declines remain uncertain, one hypothesis involves mortality from bacterial gill disease, putatively caused by Nuclear Inclusion X (NIX), a gamma proteobacterium (Kerk et al. 1992). During these events, histological evaluations revealed high prevalence (100% in 1983 and 1984, and between 60-100% in 2006-2010) and moderate intensity (between 4.6-26.7 bacteria per 40X field of view) of NIX infections in the gill epithelia, characterized by intranuclear inclusions, nuclear hypertrophy, and lysis of the cell membranes (Elston 1986, Fradkin et al. 2018).

To date, the sole diagnostic tool for assessing NIX infections is histopathological assessment of stained gill tissues. Here, we report the development of a quantitative PCR (qPCR) assay for NIX and application of this assay to wild razor clam samples from disparate geographic regions. We also developed a second, specific molecular test, based on chromogenic in situ hybridization (CISH) staining, that was used to confirm the results obtained by qPCR. These assays provide specific and sensitive methods to diagnose NIX in razor clam tissues.

2. Methods

Wild razor clams were collected by hydraulic pump, shovel, and clam gun from Kalaloch Beach, WA; North Long Beach, WA; Katmai Peninsula (along Hallo Bay), AK; and Kenai

Peninsula (between Ninilchik and Cohoe), AK (Table 1). Long Beach and Katmai peninsula were previously sampled during a 1983 survey. Kalaloch and Kenai were added to increase the number of sites. Juvenile (< 76mm) and adult (> 76mm) clams were collected, measured for length and gill tissue samples were removed aseptically and placed in 95% ethanol for qPCR or Davidson's fixative with 35‰ Instant Ocean for histology (Shaw and Battle 1957). No unusual gross pathology was noted on these clams.

Total DNA from Kalaloch Beach, WA, clams (a presumed NIX-positive population based on historical assessment) was extracted using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's instructions. The 16S ribosomal gene region of the putative NIX agent was amplified using generic bacterial primers 27F 5'-AGAGTTTGATCCTGGCTCAG-3'and 1492R 5'-GGTTACCTTGTTACGACTT-3'(Chen et al. 2015) in a PCR assay containing 50 µl of master mix (GoTaq; Promega), 1.25 units of GoTaq, 2 µM of each primer, and 1 µl of undiluted DNA extract. The cycling conditions were 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 55 °C cycles for 1 minute, 72°C for 2 minutes; and final extension of 10 minutes at 72°C in an Eppendorf 5341 PCR instrument. The expected 1400-bp amplicon was visualized in a 1.5% agarose gel stained with ethidium bromide and this amplicon subjected to Sanger sequencing using the BigDye chemistry and a 3130 genetic analyzer (Applied Biosystems) (as described by Batts et al. (2012) with 27F, 1492R, and specific NIX primers (below) for confirmation.

Quantitative PCR was run on 384 well plates in a ViiA 7 Real-Time PCR system (Applied Biosystems). The NIX 16S target sequence was aligned against closely-related proteobacterium species (GenBank accession numbers AH001812, KC169771, NR_157681, GU118891, KF179817 and KX611231) and NIX-specific primer and probe sites were identified: NIX974 forward primer, 5'-ACCTTACCTGGCCTTGACATACTGT-3'; NIX1061 reverse

primer, 5'-GCCATGCAGCACCTGTATCTGT-3'; and NIX Probe 5'-6FAM-ACT+C+GCTAGAGATAG+CT-NFQ-3' ('+' indicates locked nucleic acids) (Integrated DNA Technologies (IDT)). An artificial positive control (APC) plasmid that encodes the NIX qPCR target region was developed based on the approach of Snow et al. (2009) and allowed for NIX DNA quantification. Briefly, an artificial control that contained target sequences for the NIX primers and probe, plus the addition of an artificial probe binding site, was synthesized as a gBlock by Integrated DNA Technologies. The artificial control can be distinguished from true NIX template by incorporating a probe that binds the artificial site (as described by Snow et al. 2009); this capability was not needed during the present study because contamination was not suspected. Duplicated negative controls and serial dilutions of the APC ranging from 0.7 to 7.7 log copies per reaction were included in each qPCR reaction. The qPCR cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Duplicate reaction wells contained 12 µL of 1X ABI Universal PCR Master Mix with 900 nM of each forward and reverse primer, 200 nM concentration of probe and 5 µL DNA template. Because some but not all samples showed evidence of inhibition (quenching of fluorescent height) when we used 10 ng of total gill DNA, all samples were re-run at a 1:10 dilution (1 ng total DNA); results obtained from the diluted DNA samples are presented here. Site-level differences in infection intensity were evaluated with ANOVA using R v. 4.0.2.

Gill samples from 30 clams (10 each from Katmai, AK; Kalaloch, WA; and North Long Beach, WA) were processed by histology to confirm the presence or absence of NIX. Fixed samples were processed using standard histological procedures, sectioned to 5µm, and stained with hematoxylin and eosin (H&E) (Fischer et al. 2008). Additional sections from every clam processed for histology were processed for chromogenic in situ hybridization (CISH). CISH uses

DNA probes that hybridize to DNA in histological sections and are visible under bright-field microscopes. Methods followed the assay originally designed by Conway et al. (2015) for detection of *Ichthyophonus* sp. with the following modifications: a NIX-specific digoxygenin-labeled oligonucleotide probe (5'-5DigN/CCAAGCTATCTCTAGCGAGT-3', IDT) was diluted to 1 ng uL^{-1}; after melanin bleaching, tissues were counterstained for 30 s in CAT hematoxylin (Biocare Medical) and then soaked in Tris buffered saline (0.05M Tris, 0.14M NaCl, pH 7.6) for 1 min. Stained sections were examined microscopically at 200X magnification for the presence of NIX-like intranuclear inclusions and other pathological abnormalities. For each CISH slide, the number of inclusions was quantified for five fields of view.

3. Results

The 1417-nt NIX DNA sequence (GenBank #MN698720) was searched in NCBI BLAST and yielded a 97% identity to the R clone of uncultured gammaproteobacteria (see figure S1 for alignment with the NIX primer and probe). Other close similarities included *Parendozoicomonas haliclonae* and numerous uncultured coral reef bacteria. The new sequence had >98% nucleotide identity to the previously reported partial 16S NIX sequence (GenBank #AH001812) isolated from a Pacific razor clam collected in Copalis Beach, WA Kerk et. al (1992).

The NIX qPCR assay yielded linear amplification of the positive control standards over a 7-log range with a PCR efficiency of 94.5% (slope -3.46) and a limit of detection of 5 DNA copies per reaction. The C_T values of duplicate technical replicates were routinely consistent with mean coefficient of variation (CV) of 0.5% for the standard curve replicates and 0.8% mean CV for the gill DNA sample replicates. However, we observed evidence of PCR inhibition that

manifested as reduced fluorescent height in the raw dye traces for some reactions when using 10 ng total DNA per reaction. Diluting the DNA to 1 ng total DNA per reaction resolved this issue. NIX DNA was detected by qPCR in 100% (n = 92) of razor clams from Washington and 0% (n = 63) from Alaska (Table 1). Infection densities were significantly higher ($P < 0.001$) at Kalaloch Beach than North Long Beach, based on qPCR (1.1×10^7 and 1.4×10^6 gene copies / µg, respectively) and histology (278 and 35 intranuclear inclusions / 5 fields at 200X, respectively; Table 1).

NIX was confirmed in H&E stained and CISH histological samples from WA, but not from AK. Pathological changes associated with NIX infections included hyperplasia of the gill epithelium and nuclear inclusions. Focal areas of NIX DNA that included and extended beyond the hypertrophied nuclei were apparent in NIX-positive samples stained by CISH (Figure 1).

4. Discussion / Conclusion

Two new specific molecular assays were developed. The qPCR assay was applied as a screening diagnostic to test wild samples and the results were confirmed using CISH. Hybridization of the 16S probe to the gill nuclear inclusions confirms that the 16S sequence reported by Kerk et al. (1992) is from the NIX agent. Both assays revealed 100% prevalence of the NIX agent in selected Washington beaches but 0% infection prevalence in selected Alaskan beaches. This prevalence disparity likely reflects the geographic range for NIX; histological analysis of samples collected in 1983-1984 found 100% prevalence in clams collected on beaches in Washington, and 0% prevalence in samples collected in Haida Gwaii, BC, and on the Kenai Peninsula in Alaska (Elston et al. 1986).

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Table 1 Sample collection details and NIX results (qPCR and histology) from all locations.

Collection Site	Latitude, Longitude	Collection Date	Sample Size (n)	Mean Shell Length (mm) (±SD)	Infection Prevalence		Infection Intensity (± SD)	
					qPCR	Histology (n = 10 / site)	qPCR – Gene Copies / µg (± SD)	CISH - Intranuclear Inclusions / 5 fields @ 200X (± SD) (n = 10 / site)
N. Long Beach, WA	46.555, -124.062	5/20/19	38	21.3 (8.5)	100%	100%	1.41×10^6 ($\pm 1.20 \times 10^6$)	35 (17)
Kalaloch Beach, WA	47.612, -124.377	6/18/19	54	32.5 (9.2)	100%	100%	1.07×10^7 ($\pm 0.92 \times 10^7$)	278 (109)
Kenai Peninsula: Ninilchik to Cohoe, AK	Between 60.046, -151.683 and 60.3083, -151.385	6/16/19-6/18/19	30	31.9 (7.7)	0%	No Data	0	No Data
Katmai Peninsula: Hallo Bay, AK	58.448, -154.069	7/7/19	33	32.4 (8.5)	0%	0%*	0	0*

*NIX-like inclusions were detected in 1/10 H&E-stained samples from Katmai; however, this was a non-specific finding, as the sample was qPCR negative and did not hybridize with the CISH probe.

Figures

Figure 1. Light micrographs of sections from representative razor clam gill samples. QPCR-negative sample collected from Alaska (Katmai) and stained with H&E (A) or CISH (B). QPCR-positive sample collected from Washington (Kalaloch Beach) stained with H&E (C) or CISH (D). Arrows indicate candidates for the putative NIX agent (C), which stain heavily with CISH (D). Scale bar 50 µm.