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A POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF GENOMIC DNA OF A RICKETTSIALES-LIKE PROKARYOTE ASSOCIATED WITH WITHERING SYNDROME IN CALIFORNIA ABALONE

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ABSTRACT The 16S rDNA from a Rickettsiales-like procaryote (RLP) infecting postesophageal tissues of black abalone *Haliotis cracherodii* Leach exhibiting signs of withering syndrome (WS) was amplified, cloned, and sequenced. The 16S rDNA sequence for the RLP was similar to that of species found in the genera *Ehrlichia, Anaplasma,* and *Wolbachia.* A polymerase chain reaction (PCR) test was developed that specifically amplifies a 160 bp segment of the 16S rDNA from the RLP associated with WS. Positive reactions were obtained for all black abalone samples of digestive gland or postesophagus known to be infected with the RLP by microscopic examinations of stained tissue sections. The PCR worked equally well for infected tissues of black and red abalone *H. rufescens* Leach. There was no amplification of genomic DNA from four other microbial species isolated from cultures of intestinal flora of abalone or from abalone deemed free of the RLP by microscopic examinations. This PCR test greatly increases the ability to detect the bacterium, because to date no means to grow the organism from marine invertebrates on synthetic media or in cell lines have been developed. This PCR test should allow detection of the RLP before the onset of clinical signs of withering syndrome in cultured or wild abalone stocks. Furthermore, the test may be useful in identifying reservoirs or other related RLPs in other marine invertebrates.

KEY WORDS: Polymerase chain reaction, ribosomal DNA, Rickettsiales, abalone, *Ehrlichia, Haliotis*

INTRODUCTION

The black abalone *Haliotis cracherodii* Leach, a species once common along much of the intertidal zone of southern California's rocky beaches, has suffered severe negative impacts because of the disease termed withering syndrome (WS) (Alstatt et al. 1996., Friedman et al., 1997, Haaker et al. 1992, Tissot et al. 1991, VanBlaricom et al., 1993, 1996). Affected populations of black abalone have been reduced to 1–10% of population densities observed before the onset of WS (Haaker et al. 1992). The disease has now been observed among farmed red abalone (*H. rufescens*) (Moore et al. in press), and this has prompted the California Department of Fish and Game to place a partial ban on movement of cultured red abalone from locations where WS is endemic to locations free of this disease. The epizootic manner in which the disease has spread throughout black abalone in the Channel Islands and the mainland indicate a role for an infectious etiologic agent (Lafferty and Kuris 1993). Although not completely proved, evidence suggests that WS is caused by a previously undescribed Rickettsiales-like prokaryote (RLP), which infects gastrointestinal epithelia (Friedman et al. 1997, Gardner et al. 1995). Withering syndrome and the associated RLP have also been documented in wild and cultured red abalone, *Haliotis rufescens* Leach, (Haaker et al. 1992, Moore et al. in press). Because of the inability to culture most marine RLPs, differentiation of these organisms is difficult and is based upon morphological characteristics using light and electron microscopy. Sensitive and accurate detection of the WS-associated RLP is critical to our understanding and control of the spread of WS. The goal of the current study was to develop a polymerase chain reaction (PCR) test to improve our detection and understanding of the biology of the RLP causing WS in abalone and other as yet unidentified hosts.

MATERIALS AND METHODS

DNA Extractions, Sequencing, and Alignments of rDNA

DNA isolation from whole tissue and from bacterial cells was performed as described by Sambrook et al. (1989) and Friedman et al. (in press). Briefly, rinsed tissues were homogenized in lysis/proteinase K buffer. After 1 h at 55 °C, the DNA was extracted in a phenol-chloroform solution. Isoamyl alcohol was added, mixed for 10 min, and centrifuged. The top aqueous phase was removed and 1/10 volume of 3 M sodium acetate was added. Cold absolute ethanol was added to precipitate the DNA. The pellet was washed in 70 % ethanol, air-dried, and resuspended in Tris-EDTA (TE) buffer. Alternatively, DNA was prepared using the QIAmp Tissue Kit (Qiagen Inc., Valencia, CA) following the manufacturers "mouse tail protocol". Bacterial 16S rDNA from infected abalone tissue was amplified using EUB A and EUB B universal eubacterial 16S rDNA primers (Giovannoni 1991). The RLP 16S rDNA gene was cloned into pCR2.1 using a TOPO Cloning Kit (Invitrogen, San Diego, CA) following manufacturer's protocols. Clones were screened using PCR with primers that flanked the multicloning site of the vector. Positive clones were selected from among those that had an insert of the appropriate size (~1550 bp). The 16S rDNA sequencing and alignments of sequences were performed as described previously (Andree et al. 1997). The completed sequence was used in a BLAST search of GenBank to confirm the similarity of the sequence to other Rickettsiales. The species appearing in the results of the BLAST search were compared with other closely related bacterial species in a pairwise analysis of sequence similarity (Table 1) in addition to a phylogenetic distance analysis (Friedman et al. in press).

TABLE 1.

A pairwise comparison of the 16S ribosomal DNA sequence similarity seen among closely related species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 WFS-RLP		74.0	73.3	73.2	77.3	75.4	75.5	75.8	71.3	69.7	73.4	73.3	70.9	69.8	69.0
2 *W. pipientis*			77.8	77.6	80.7	80.5	81.5	81.0	74.1	73.6	77.3	77.5	73.7	73.3	73.0
3 *E. sennetsu*				94.5	78.4	77.9	79.7	78.9	74.7	73.5	76.3	76.6	74.2	72.6	71.3
4 *E. risticii*					78.2	77.5	79.5	78.7	74.9	73.5	76.0	76.4	74.3	73.3	71.0
5 *A. marginale*						85.8	89.4	88.2	76.9	75.1	77.1	77.6	75.9	73.6	73.7
6 *C. ruminantium*							86.2	85.4	76.7	73.8	77.5	77.8	74.1	72.7	71.5
7 *E. phagocytophila*								91.2	76.8	74.3	78.3	78.5	75.7	74.0	73.1
8 *E. bovis*									76.1	74.1	77.6	77.6	75.6	73.2	72.9
9 *C. caryophila*										74.5	77.3	77.8	78.8	74.6	72.3
10 *C. burnettii*											74.4	75.0	72.5	80.5	78.1
11 *R. prowesekii*												93.0	76.7	74.3	71.8
12 *R. rickettsii*													77.1	74.3	71.8
13 NHP														72.3	70.3
14 *P. salmonis*															81.5
15 *E. coli*															

Primer Selection and Oligonucleotide Synthesis

A BLAST search of GenBank indicated three species, *Anaplasma marginales, Ehrlichia bovis*, and *Wolbachia pipientis*, were most similar to the 16S rDNA sequence amplified from infected abalone (clone designation p16RK3; GenBank accession number: AF133090). The four sequences above and that of *Piscirickettsia salmonis* and a Rickettsiales-like prokaryote from shrimp (Frelier et al. 1993) were aligned to identify those sequences most unique to the RLP from infected abalone. Several primers were selected for synthesis and testing. In an initial trial, two primers designated RA5-1 [$^{5\prime}$GTTGAACGTGCCTTCAGTTTAC$^{3\prime}$] and RA3-1 [$^{5\prime}$CTGAGGCCATCTGTTAAAATGG$^{3\prime}$], were synthesized (Gibco BRL, Inc., Gaithersburg, MD) and used in an initial screening of samples containing enriched RLP from abalone tissues. The best results were obtained using these primers in conjunction with an annealing temperature of 55 °C. An amplified product of 946 bp was obtained from all RLP-enriched samples tested (data not shown). Subsequent tests with DNA samples extracted from digestive gland tissues of diseased animals showed a poor correlation with the histology results (e.g., samples from known positive abalone were negative by PCR). We speculated that the 16S rDNA of the normal gut flora could be hybridizing with the primers for the PCR, reducing efficiency of amplification. Accordingly, we designed new primers for the WS-PCR based on a second alignment using the 16S rDNA sequences from p16RK3, *E. bovis, A. marginales, W. pipientis, P. salmonis,* and *E. coli* as a representative of intestinal flora. This alignment showed the sequence of primer RA3-1 to be completely conserved in the 16S rDNA of *E. coli*, and this could have been contributing to the poor results observed in the PCR by nonspecific hybridization of this primer to bacterial DNA in the sample. We, therefore, designed three additional primers designated RA5-6 [$^{5\prime}$GAAGCAATATTGTGAGATAAAGCA$^{3\prime}$], RA3-6 [$^{5\prime}$ACTTGGACTCATTCAAAAGCGGA$^{3\prime}$], RA3-8 [$^{5\prime}$CCACTGTGAGTGGTTATCTCCTG$^{3\prime}$] for testing as potential primers for the WS-PCR. The primers RA5-1 and RA3-6 were designed to amplify a sequence of ~160 bp from the 5′ end of the 16S rDNA (Fig. 1). The primer pair RA5-6 and RA3-8 was designed to amplify a sequence of ~230 bp from the 3′ end of the 16S rDNA (Fig. 1). The previously extracted DNA samples were then retested with these new primer sets.

We also examined the possibility of using crude cell lysates rather than purified DNA in the assays for the WS-PCR. Tissue samples from 26 abalone were prepared by homogenization in TE buffer and boiling for 5 minutes at 100 °C. From this solution, 3 µl was added to a PCR cocktail. After amplification, DNA was separated on 1.5 % agarose gels.

PCR Amplification of rDNA

All amplifications were performed in standard 50 µl reactions containing 10 mM Tris-HCl pH 8.3 (at 25 °C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % w/v gelatin, 400 µM dNTPs, 5 µM tetramethyl ammonium chloride, 40 pmoles of each primer, and 2 U Taq polymerase. The PCR thermal cycler used was a model PTC-200 (MJ Research, Watertown, MA).

A 160 bp fragment (using primers RA5-1 and RA3-6) of the 16S rDNA from the RLP was amplified using 40 cycles of 1 min at 95 °C, followed by 30 sec at 50 °C, followed by 30 sec at 72 °C. The amplification cycles were preceded by a denaturation step, where samples were held at 95 °C for 5 min and followed by an extended elongation step where samples were held at 72 °C for 10 min.

Specificity of the PCR

Adult black abalone were collected from the Vandenberg Air Force Base, CA (in July 1996) where WS is epidemic and from Monterey, CA (in December 1998) where the RLP has been recently detected but where no signs of clinical WS have yet become apparent (Finley and Friedman, unpubl. obs). Additional samples of adult abalone were collected from the following two locations where neither WS nor the RLP had been observed: black abalone from Carmel Point in August 1997 and red abalone from Shelter Cove in December 1998. Farmed red abalone obtained in January 1999 from a facility in Central California were also examined. Visual assessment of WS was determined according to Friedman et al. (1997). Digestive gland, postesophagus and/or epipodium were collected and stored at −80 °C until DNA extraction. The hemolymph of abalone contains no blood-clotting factor; there-

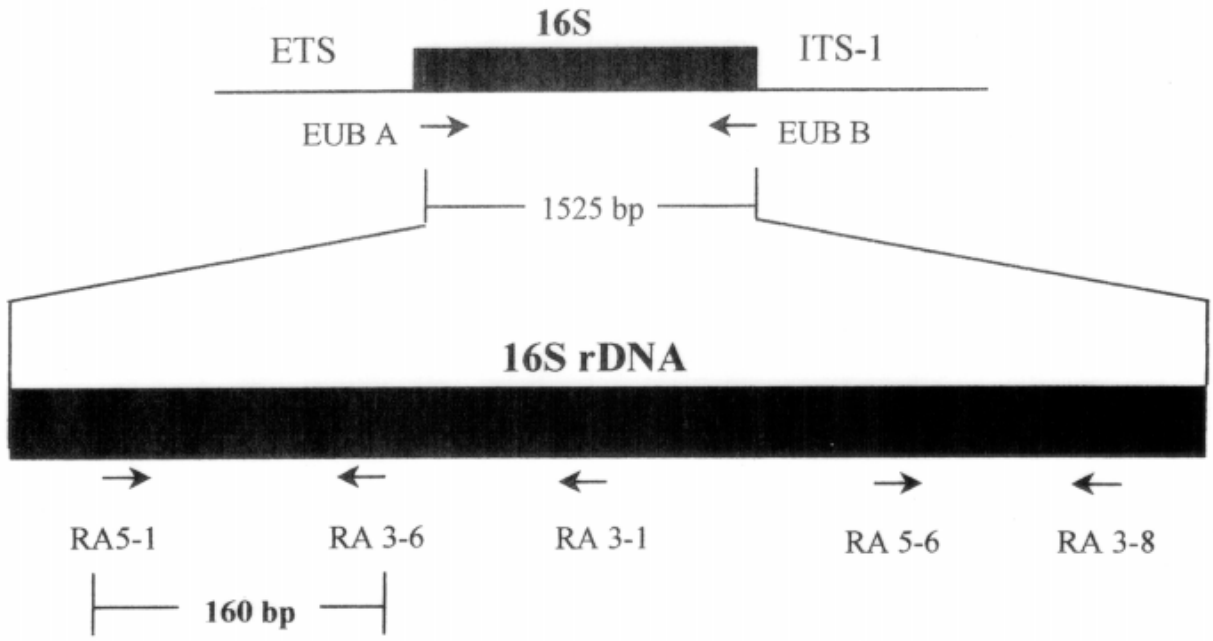

Figure. 1. Diagrammatic representation of the approximate location and orientation of primers used for development of the PCR assay to detect the Rickettsial-like prokaryote among abalone with withering syndrome (WS). Primers EUB A and EUB B were used in the initial amplification of the 16S rDNA from infected tissues.

fore, collection of samples from internal organs is fatal for the animal. DNA samples obtained from different abalone tissues were tested to identify those best for use in PCR assays and to determine if the RLP could be detected in nonlethal samples of the epipodium.

Genomic DNA of endogenous gut flora was tested for possible nonspecific amplification of DNA using the WS diagnostic primers. To obtain cultures for DNA extraction, postesophagus tissue (0.8–2.0gm) was dissected from three black abalone (Vandenberg Air Force Base, CA) and held separately in sterile vials containing 10 mL of 0.2 μm-filtered seawater, on ice. Vials were shaken vigorously to dislodge and suspend bacterial flora associated with epithelial surfaces, and the tissue was removed from each vial. The bacterial suspensions were spread onto marine and TCBS agar plates that were held at 15 °C. Colonies appearing after 7 days were streaked on marine agar plates. Four colonies from the TCBS plates and two from the marine agar plates were selected and passed several times on marine agar. Based on biochemical (API NFT Biomerieux Vitek, Hazelwood, MO) tests, supplemental tests, and colony morphology on TCBS and marine agar, four distinct isolates were obtained (Table 2). Cultures grown in TSB broth (supplemented with 2 % additional NaCl) were centrifuged (3,200 *g*, 10 min, 4 °C), resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA), and frozen at –80 °C. The presence of any RLPs among the colonies tested was precluded by the multiple passages of the isolates on artificial media. DNA was extracted from the bacterial cultures, as described above. Approximately 150 ng of genomic DNA from each of these isolates was tested with the primers RA5-1 and RA3-6.

Histology

Several 3-mm cross sections that included mantle, epipodium, postesophagus, digestive gland, and foot muscle were excised from each abalone, placed in Invertebrate Davidson's solution (Shaw and Battle 1957) for 24 h and processed for routine paraffin histology. Deparaffinized 5 μm tissue sections were stained with Harris's hematoxylin and eosin (Luna 1968) and assessed for the presence of RLPs, and condition of the foot muscle and digestive gland were evaluated (Friedman et al. 1997). The digestive gland was scored as 0 if normal, with terminal acini occupying most of the tissue present. A score of 1 represented a moderate degeneration of or replacement of terminal acini with transport ducts or connective tissues; whereas, a score of 2 represented a severe loss of acini. Similarly, condition of the foot muscle was scored as 0 when muscle bundles were tightly packed, 1 when a moderate loss

TABLE 2.

Characteristics of four postesophagous bacterial isolates grown in culture and tested for reactivity using the WS-RLP PCR protocol.

Isolate	Gram	Shape	Sucrose Reaction on TCBS	O/129 Sensitivity	H2S Production	PCR Reaction
150-A	Negative	Rods	Positive	Sensitive	Negative	Negative
150-B	Negative	Rods	Negative	Sensitive	Negative	Negative
213	Negative	Rods	Negative	Resistant	Positive	Negative
239	Negative	Rods	Negative	Resistant	Negative	Negative

of muscle bundles and concomitant increase in connective tissue was observed, and 2 when such loss was severe.

RESULTS

PCR Detection of the Putative Etiologic Agent of Withering Syndrome

The DNA from tissues of abalone with naturally acquired infections was screened for the presence of RLP DNA. Tissues from abalone collected from geographic regions where signs of WS and the associated RLP were absent served as negative controls. In addition, epipodial tissue was tested to determine if nonlethal samples could serve as diagnostic material for PCR analysis.

In total, we examined 23 animals by PCR (Table 3). We compared epipodium, digestive gland, and/or postesophagus for some individual animals. The 160 bp amplicon was present only in tissues from those animals that came from WS enzootic areas (most of which had proved to be positive by microscopic examination). The identity of the amplified DNA was confirmed by automated sequencing (data not shown). In addition, the identity of the species being detected was confirmed by *in situ* hybridization experiments in which the PCR primers were used as probes (Antonio et. al. in press). The yield of amplified DNA was greatest from postesophageal tissue (Fig. 2). This agrees with microscopic observations that demonstrated the postesophagus was more highly infected than the digestive gland. A weak amplification of DNA was obtained from some samples of epipodium from a subset of animals that tested strongly positive using the digestive gland.

Specificity of the PCR

Of the primer pairs tested, RA5-1 and RA3-6 gave the best results at an annealing temperature of 50 °C. Retesting of samples with this new primer produced a 160 bp amplicon from all tissues known to be positive for the RLP by microscopic examinations. In addition, all samples considered free of the RLP by microscopic examination were negative by PCR. There was no amplification of genomic DNA from the selected bacterial isolates from the intestine of black abalone or from a recently isolated *Piscirickettsia*-like organism isolated from white sea bass *Atractoscion nobilis* Ayres in California (unpubl. obs.). Only DNA extracted from abalone tissues known to contain RLP yielded a 160 bp amplicon. All samples of postesophagus and digestive gland from RLP-infected red and black abalone tested positive. There was a 250 bp amplicon present from the epipodial tissue of six black abalone collected from Vandenberg, Monterey, and Shelter Cove. However, two of these six samples yielded both amplicons (160 bp and 250 bp). Those with only the 250 bp amplicon were scored as negative based on the difference in the molecular weight and sequencing of the larger amplicon that indicated it was not related to bacterial 16S rDNA.

DISCUSSION

Diagnosis of infectious disease during the past century has generally relied on such methods as culture, direct observation of parasites or, more recently, antigen-based assays (Sethi et al. 1996). These methods may involve expense and time for sample

TABLE 3.

Detection of a Rickettsiales-like prokaryote by PCR and histology in digestive gland, postesophagous and epipodium tissues in black and red abalone in various stages of withering syndrome.

Animal #	Species	Source/Date	WS sign[a]	Digestive gland PCR[b]	Digestive gland Histology[c]	Postesophagus PCR[b]	Postesophagus Histology[c]	Epipodium PCR[b]	Epipodium Histology
1	Black	Carmel Pt. 8-97	0	–	0	nd	0	–	–
2	Black	Carmel Pt. 8-97	0	–	0	nd	0	–	–
3	Black	Carmel Pt. 8-97	0	–	0	nd	0	–	–
4	Black	Carmel Pt. 8-97	0	–	0	nd	0	–	–
5	Red	Monterey 12-98	0	–	0	nd	0	–	–
6	Red	Monterey 12-98	0	–	0	nd	0	–	–
7	Red	Monterey 12-98	0	–	0	nd	0	–	–
8	Red	Shelter Cove 12-98	0	–	0	nd	0	–	–
9	Red	Shelter Cove 12-98	0	–	0	nd	0	–	–
10	Red	Shelter Cove 12-98	0	–	0	nd	0	–	–
11	Black	Monterey 12-98	0	++	1	nd	1	+	–
12	Black	Monterey 12-98	0	++	1	nd	1	–	–
13	Black	Monterey 12-98	0	++	2	nd	2	–	–
14	Black	Vandenberg 1997	0	++	1	nd	2	–	–
15	Black	Vandenberg 1997	1	+	0	nd	2	+	–
16	Black	Vandenberg 1997	2	++	2	nd	3	+	–
17	Black	Vandenberg 1997	2	+++	3	nd	3	+	–
18	Red	Farm A 1-99	2	+++	2	nd	2	–	–
19	Black	Vandenberg 1997	3	–	0	+++	3	nd	–
20	Black	Vandenberg 1997	3	++	3	+++	3	nd	–
21	Black	Vandenberg 1997	3	+++	3	nd	3	++	–
22	Red	Farm A 1-99	3	+++	2	++++	3	–	–
23	Red	Farm A 1-99	3	+++	3	nd	2	–	–

[a] WE sign: Degree of body mass shrinkage (0 = within normal range, 1, 2, 3 = slightly, moderately, severely shrunken).

[b] Relative intensity of 160 bp amplicon band in ethidium bromide stained gels (– = absent, ++++ = brightest, nd = no data).

[c] RLP infection intensity by microscopic examination (0 = absent, 1 = low density, 2 = moderate, 3 = high).

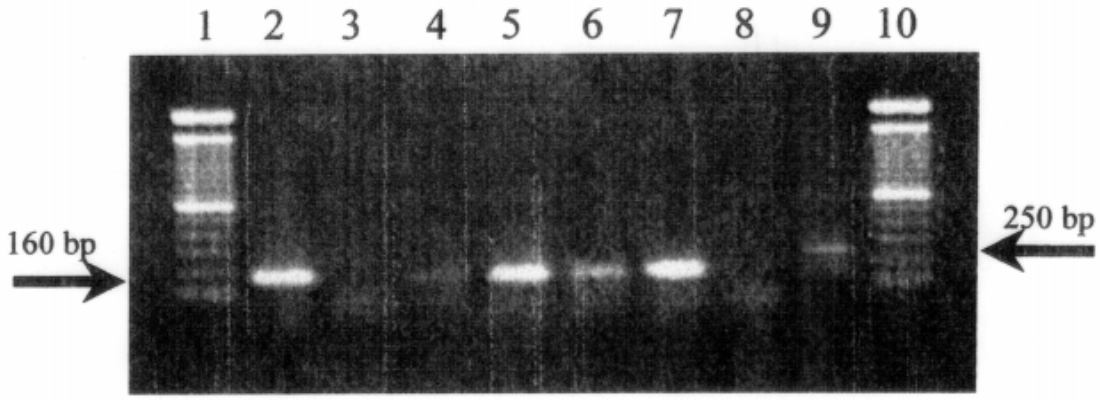

Figure. 2. Detection of a Rickettsial-like prokaryote in tissues from abalone with withering syndrome (WS) using a newly developed PCR assay. Approximately 300 ng of genomic DNA was used for each sample assay. Lanes 1and 10: 100 bp ladder molecular weight standard; lane 2: positive control sample (10 ng of plasmid p16RK3 containing cloned 16S rDNA); lane 3: negative control sample (uninfected black abalone postesophagus tissue); lanes 4 and 5, respectively: digestive gland and postesophagus of infected black abalone; lanes 6 and 7, respectively: digestive gland and postesophagus of infected red abalone; lane 8: negative control sample (150 ng of genomic DNA from prokaryote cultured from the gut of abalone); lane 9: epipodial tissue from infected black abalone displaying the 250 bp amplicon.

preparation or may lack sensitivity in detecting low numbers of parasites. Furthermore, many parasites, including the RLP from abalone, have not been cultured outside the host animal (Arnoldi et al. 1992, Mari et al. 1995, Sethi et al. 1996). Molecular approaches to parasite detection such as the PCR are rapid, reproducible, and relatively easy to conduct (Andree et al. 1998, Mauel et al. 1996, Sethi et al. 1996). Early and accurate detection of parasites in marine invertebrates is critical, because therapeutic approaches are limited, and avoidance becomes a principal means of disease management and resource protection.

Withering syndrome is a fatal, infectious, bacterial disease of both wild and cultured abalone in California (Friedman et al. 1997). Diagnosis of WS, like many diseases, currently relies on observation of gross signs of affected abalone and detection of the RLP in target tissues by microscopic examination of stained tissue sections. Unfortunately, inclusions of other intracellular bacteria are frequently found in shellfish and are often morphologically indistinguishable from the RLP associated with WS (Friedman and Hedrick, unpubl. obs.). We have confirmed the PCR test differentiates among RLPs in abalone tissues by *in situ* hybridization experiments that utilize the PCR primers as probes (Antonio et al. in press). The probes only hybridize to the RLPs that correlate with the observation of disease symptoms (data not shown). The RLP is, therefore, more easily and rapidly detected by the application of such DNA diagnostic tests as PCR than by microscopy.

This PCR test should prove to be a useful tool for the study of WS, especially for epidemiological investigations of the modes of transmission and reservoirs for the parasite in the marine habitat. In addition, experimental exposure studies combined with this PCR assay should help to identify naturally resistant populations of black abalone. The PCR assay provides direct visualization of specific bands on ethidium bromide stained gels, more rapid diagnosis than microscopic examination of tissue sections, and obviates the need for cell culture isolation of the parasite. Furthermore, we presume the PCR test will detect organisms at lower levels of infection than microscopic examination of stained tissue sections.

The primers designed for PCR detection of the RLP were chosen after alignments showed sequences obtained from *Anaplasma marginales, Ehrlichia bovis, Wolbachia pipientis, Piscirickettsia salmonis, Escherichia coli*, and an unnamed Rickettsia-like bacterium from shrimp found no homologous sites for hybridization. The lack of hydridization of the RLP primers with genomic DNA isolated from bacterial flora cultured from the postesophagus of abalone and from *P. salmonis* genomic DNA from cells grown in tissue culture was indicated by the absence of any amplified products following the PCR. The assay performed equally well on RLP-infected black and red abalone. There was some nonspecific amplification from epipodial tissues of a small number of the black abalone tested but the product (250 bp) was clearly different in molecular weight and sequence from the expected 160 bp amplicon. This 250 bp product may be the result of surface contamination of the epipodium, because it was not observed in red abalone, or black abalone from all locations.

Some heavily infected animals gave positive test results with epipodial tissues; however, in general, most samples of the epipodium were negative by PCR. The relatively weak positives among the epipodium samples may represent cross contamination (with more heavily infected tissues) during sampling or the adherence of RLPs on the epipodium as shed from infected animals in crowded tanks during transport or holding before sample collection. This concern combined with the appearance of the 250 bp amplicon in nonlethal epipodial biopsies discourages us from recommending this sampling approach for detection of the RLP. A more reliable approach is to collect postesophagus or digestive gland tissues from each animal, which requires sacrificing the animal (as do current microscopic procedures).

As mentioned above, an additional application of the PCR primers is their use for *in situ* hybridization (ISH) to visualize the parasite in various tissues or alternate hosts and to differentiate this bacterium from other RLPs commonly observed in marine species (Elston 1986, LeGall et al. 1988). Future work utilizing ISH may also identify portals of entry and early developmental forms not easily seen by standard microscopic examinations, as shown with other parasites of aquatic hosts (Antonio et al. 1999; Antonio et al. in press).

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LITERATURE CITED

Andree K. B., S. J. Gresoviac & R. P. Hedrick. 1997. Small subunit ribosomal RNA sequences unite alternate actinosporean and myxosporean stages of *Myxobolus cerebralis* the causative agent of whirling disease in salmonid fish. *J. Eukaryot. Microbiol.* 44:208–215.

Andree K. B., E. MacConnell & R. P. Hedrick. 1998. A nested polymerase chain reaction for the detection of genomic DNA of *Myxobolus cerebralis* in rainbow trout (*Oncorhychus mykiss*). *Dis. of Aquat. Org.* 34:145–154.

Antonio D. B., K. B. Andree, T. S. McDowell & R. P. Hedrick. 1999. Detection of *M. cerebralis* in rainbow trout (*Oncorhynchus mykiss*) and oligochaete tissues using a nonradioactive *in situ* hybridization protocol. *J. Aquat. Anim. Health* 10:338–347.

Antonio D. B., K. B. Andree, J. D. Moore, C. S. Friedman & R. P. Hedric. in press. Detection of Rickettsiales-like Prokaryotes (RLPs) by *in situ* hybridization in black abalone *Haliotis cracherodii* with withering syndrome. *J. Invert Path.*

Altstatt, J. M., R. F. Ambrose, J. M. Engle, P. L. Haaker, K. Lafferty, & A. Kuris. 1996. Recent declines of black abalone *Haliotis cracherodii* on the mainland coast of central California. *Mar. Ecol. Prog. Ser.* 142: 185–192.

Arnoldi, J., C. Schluter, M. Duchrow, L. Hubner, M. Ernst, A. Teske, H. D. Flad, J. Gerdes & E. C. Bottger. 1992. Species-specific assessment of *Mycobacterium leprae* in skin biopsies by *in situ* hybridization and polymerase chain reaction. *Lab. Invest.* 66:618–623.

Elston, R. 1986. Occurrence of branchial rickettsiales-like infections in two bivalve mollusks, *Tapes japonica* and *Patinopecten yessoensis*, with comments on their significance. *J. Fish Dis.* 9:69–71.

Friedman, C. S., K. B. Andree, K. Beauchamp, J. Moore, J. D. Shields, & R. P. Hedrick. in press. *Xenohaliotis californiensis* gen. nov., sp. nov., a pathogen of abalone, *Haliotis* spp., along the west coast of North America. *Int. J. Syst. Bacteriol.*

Friedman, C. S., M. Thomson, C. Chun, P. L. Haaker & R. P. Hedrick. 1997. Withering syndrome of the black abalone, *Haliotis cracherodii* (Leach): water temperature, food availability, and parasites as possible causes. *J. Shellfish Res.* 16:403–411.

Frelier, P. F., J. K. Loy, & B. Kruppenbach. 1993. Transmission of necrotizing hepatopancreatitis in *Penaeus vannamei*. *J. Invertebr. Pathol.* 61:44–48.

Fryer, J. L. & C. N. Lannan. 1994. Rickettsial and chlamydial infections of freshwater and marine fishes, bivalves, and crustaceans. *Zool. Stud.* 33:95–107.

Fryer, J. L., C. N. Lannan, S. J.Giovannoni & N. D. Wood. 1992. *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. *Int. J. Syst. Bacteriol.* 42:120–126.

Gardner, G. R., J. C. Harsbarger, J. L. Lake, T. K. Sawyer, K. L. Price, M. D. Stephenson, P.L. Haaker & H. A.Togstad. 1995. Association of prokaryotes with symptomatic appearance of withering syndrome in black abalone *Haliotis cracherodii*. *J. Invertebr. Pathol.* 66:111–120.

Giovannoni, S. 1991. The polymerase chain reaction. pp. 177–201. *In:* E. Stackebrandt & M. Goodfellow (eds.). Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, New York.

Haaker, P. L., D. V. Richards, C. S. Friedman, G. E. Davis, D. O. Parker & H. A. Togstad. 1992. Mass mortality and withering syndrome in black abalone, *Haliotis cracherodii*, in California. pp. 214–24. *In:* S. A. Shepherd, M. J. Tegner, and P. Guzmán del Próo, S. A. (eds.). Abalone of the World. Biology, Fisheries, and Culture. Fishing News Books, Blackwell Scientific, Oxford, England.

Lafferty, K. D. & A. K. Kuris. 1993. Mass mortality of abalone, *Haliotis cracherodii*, on the California Channel Islands: tests of epidemiological hypotheses. *Mar. Ecol. Prog. Ser.* 96:239–248.

LeGall, G., D. Chagot, E. Mialhe & H. Grizel. 1988. Branchial rickettsiales-like infection associated with a mass mortality of sea scallop *Pecten maximus*. *Dis. Aquat. Org.* 4:229–232.

Loy, J. K., P. F. Frelier, P. Varner & J. W. Templeton. 1996. Detection of the etiological agent of necrotizing hepatopancreatitis in cultured *Penaeus vannamei* from Texas and Peru by polymerase chain reaction. *Dis. Aquat. Org.* 25:117–122.

Luna, L. G. (ed.). 1968. Manual of histologic staining methods of the armed forces institute of pathology, 3rd ed., McGraw-Hill, New York. pp. 38–39.

Mari, J., D. V. Lightner, B. T. Poulous & J. R. Bonami. 1995. Partial cloning of the genome of an unusual shrimp parvovirus (HPV): use of gene probes in disease diagnosis. *Dis. Aquat. Org.* 22:129–134.

Mauel M. J., S. J. Giovanni & J. L. Fryer. 1996. Development of polymerase chain reaction assays for detection, identification, and differentiation of *Piscirickettsia salmonis*. *Dis of Aquat Org.* 26:189–195.

Moore J. D., T. T. Robbins & C. S. Friedman. in press. Withering syndrome in farmed red abalone Haliotis rufescens: thermal induction and association with a gastrointestinal Rickettsiales-like prokaryote. *J. Aquat. Anim. Health.*

Sambrook, J., E. F. Fritsch & Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Springs Harbor Laboratory, New York.

Sethi, S., T. F. Murphy & K. L. Klingman. 1996. Diagnosis, epidemiology, and pathogenesis of bacterial infections in the molecular era. *Clin. Molec. Pathol.* 49:M1–M7.

Shaw, B. L. & H. I. Battle. 1957. The gross and microscopic anatomy of the digestive tract of the oyster, *Crassostrea virginica* (Gmelin). *Can. J. Zool* 35:325–347.

Tissot, B. N., J. Lubchenco & S. Naravette. 1991. Effects of global warming on coastal marine ecosystems. implications of thermal discharge studies. *Bull. Ecol. Soc. Am.* 72:268.

VanBlaricom, G., J. Ruediger, D. Woodard, C. S. Friedman & R. P. Hedrick. 1993. Symptomatic appearance of withering syndrome at San Nicholas Island. *J. Shellfish Res.* 12:185–188.