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PERKINSUS SP. INFECTION RISK FOR MANILA CLAMS, VENERUPIS PHILIPPINARUM (A. ADAMS AND REEVE, 1850) ON THE PACIFIC COAST OF NORTH AND CENTRAL AMERICA

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ABSTRACT: Manila clams (Venerupis philippinarum, A. Adams and Reeve 1850) are an important aquaculture species on the west coast of North America and are also cultured in Europe, Asia, and other locations. Clams cultured on the west coast of North America are free of Perkinsus sp. infections, while clams from certain Asian and European sources are infected. Infection in Korean Manila clams is reportedly associated with high morbidity and mortality. We evaluated the health status of readily accessible Manila clams from Korea that were proposed for importation into Mexican waters where they would increase in size, and then be shipped into the United States, either to market destinations or to receiving waters. The examination of the clams was performed as a preliminary assessment for a producer considering the importation of Korean Manila clams. We report finding a high prevalence of a Perkinsus sp. causing significant tissue damage in juvenile Korean Manila clams. Parasite taxonomic verification was made using a genus-Perkinsus SSUrRNA gene-specific DNA probe for in situ hybridization. The use of this probe is validated and reported for the first time. As a result of this finding, no importation of this clam stock took place. It is urgently important to make widely known the risk of the spread of this disease into the clam stocks of the west coast of North and Central America to prevent such an introduction. In addition, we report new information regarding the prevalence and intensity of this disease in juvenile clams available for export, as well as pathologic features of the disease.

KEY WORDS: Venerupis (Tapes) philippinarum, juvenile clam infection, Perkinsus sp., DNA probe, in situ hybridization

INTRODUCTION

Manila clams (Venerupis philippinarum, A. Adams and Reeve 1850) are an important aquaculture species on the west coast of North America. More than 7 million pounds of littleneck clams, predominantly V. philippinarum, were produced in Washington, California, and Oregon in 2000 (Pacific Coast Shellfish Growers Association 2003), and additional production occurs in British Columbia, Canada. Although Alaska produces native littleneck clams, Protothaca staminea (Conrad 1837), Manila clams are exotic, and importation for aquaculture purposes is prohibited. Venerupis philippinarum is also an important aquaculture species in Europe and Asia, and is infected with Perkinsus sp. on both continents. Specifically, Perkinsus atlanticus occurs in Europe (Navas et al. 1992), a P. atlanticus-like parasite occurs in Japan (Hamaguchi et al. 1998), and Perkinsus sp. occurs in Korea (Choi & Park 1997) and China (Liang et al. 2001). Consistent with the close homology noted between DNA sequences at several P. atlanticus and Perkinsus olseni loci by diverse investigators, Murrell et al. (2002) assert these parasitic species to be synonymous, with taxonomic priority to the P. olseni name.

In contrast, clams from the west coast of North America are free of Perkinsus sp. infections. A survey of Manila clam health and conditions on the west coast of North America (Pacific Shellfish Institute 2001), and the required examination of over 3000 clams for health certifications from 1991 to 2002, showed no evidence of Perkinsus sp. infection. Moreover, such infections have not been reported elsewhere on the west coast during routine annual examinations and frequent health examinations of brood stocks and seed clams since 1985. In addition, Perkinsus sp. infection has not been reported in the native littleneck clam P. staminea or any other bivalve species from the west coasts of North or Central America.

Manila clams may be imported as a live market product from Korea, Japan, or other Asian countries into North America. In 1998, we evaluated the health status of juvenile Manila clams from Korea that had been proposed for importation into Mexican waters, where they would gain size before shipment to the United States, either to market destinations or to receiving waters for further growth. The examination of clams was performed as a preliminary assessment for a producer considering the importation of Korean Manila clams. We report the finding of a high prevalence of a Perkinsus sp. causing significant tissue damage in juvenile Korean Manila clams.

As a result of this finding, no importation of this clam stock took place. It is urgently important to make widely known the risk of the spread of this disease to west coast North American clam stocks to prevent the introduction of this debilitating and lethal clam parasite. In addition, we report here new information regarding the prevalence and intensity of this disease in juvenile clams that are available for export, as well as pathologic features of the disease. Finally, a novel genus-Perkinsus DNA probe for in situ hybridization (ISH) assays on histologic samples is described.

Taxonomic references to the Manila clam (also commonly referred to as the Japanese littleneck clam) in the scientific literature are particularly confusing. We have designated the species as V. philippinarum in accordance with the Committee on Scientific and Vernacular Names of Molluscs within the Council of Systematic Malacologists, American Malacological Union (American Fisheries Society 1998). The common name Manila clam is also found in the literature, apparently in reference to the same species, associated with scientific designations of Tapes philippinarum, Ruditapes philippinarum, Tapes semidecussatus, and Tapes japonica.

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 MATERIALS AND METHODS

A total of 64 Manila clams [16–32 mm shell length (SL)] from Incheon Bay, South Korea, were clinically examined in February 1998 and were fixed whole in Davidson’s shellfish fixative (Shaw & Battle 1957). These tissues were processed for routine histologic examination.

A representative tissue section containing parasites was evaluated by ISH. The genus-Perkinsus DNA probe was designed to specifically target SSU rRNA sequences of Perkinsus species by aligning the available SSU rRNA gene sequences, while not hybridizing to the sequences of closely related parasite taxa including dinoflagellates and apicomplexans. An SSU rRNA gene sequence is not available for Perkinsus sp. The resulting probe Perksp700DIG (5'-CGCACAGTTAGTTCGGRGCACG-3') was 5' end-labeled with digoxigenin (Sigma-Genosys, The Woodlands, TX). ISH assays were performed as previously described (Stokes & Burreson 1995, Stokes & Burreson 2001), except that 125 µg/mL pronase was used for permeabilization, instead of protease K, for a 30-min digestion, and a probe concentration of 7 ng/µL was used for hybridization. The probe was tested on an array of Perkinsus sp.-infected, paraffin-embedded tissues (Table 1), including Perkinsus marinus in Crassostrea virginica, P. atlanticus in Ruditapes decussatus, P. olseni in Haliothis laevigata, Perkinsus andrewsi in Macoma balthica, Perkinsus sp. in Venerupis philippinarum from Japan, Perkinsus chesapeakei in Mya arenaria, Perkinsus mediterraneus n. sp. in Ostrea edulis (Casas et al. in press), Perkinsus sp. in Chama pacifica, and P. quagwadi in Patinopecten yessoensis. Probe specificity was validated by testing tissue sections of the blue crab Callinectes sapidus, which was infected with the parasitic dinoflagellate Hematodinium sp. (Shields 1994), Hematodinium sp.-infected Norwegian lobster Nephrops norvegicus (Field & Appleton 1995), Haplosporidium nelsoni-infected and Haplosporidium costae-infected C. virginica oysters, and spot prawn Pandalus platyceros, infected by an undescribed haplosporidan-like protozoan parasite (Bower & Meyer 2002). Replicate sections of nonspecific ISH assay signal controls of each sample were tested identically, except that they received hybridization buffer without probe during the overnight hybridization step.

RESULTS

Histologic Evaluation of Infected Clams

The prevalence of juvenile clams infected with the presumptive Perkinsus sp., was 59 of 64 (92%), based on histologic examination. The protozoa were systemically distributed in a variety of organs, most typically in subepithelial areas of the gills, and fre-
quently in the mantle and labial palps. Parasites were often associated with tissue hemocytosis (Fig. 1) and occurred as single or multiple trophozoites (Fig. 2). In severe infections, the parasites were more abundantly distributed in the tissues, including the vascular sinuses around the digestive diverticula. Broad areas of the subepithelial connective tissues were composed of solid masses of parasite cysts in the most severe infections. In many cases, the parasites were contained within a thin-walled cyst formed by one to several host cells (Fig. 3). Such encapsulations contained up to 10 protozoan cells and associated hemocytosis. The parasites were often characterized by the presence of an eccentric vacuole (Fig. 1 and 3), characteristic of Perkinsus sp. trophozoites.

Confirmation of Perkinsus sp. by ISH

The genus-Perkinsus SSU rRNA gene probe Perksp700DIG demonstrated strong hybridization to Perkinsus sp. cells in all of the tissue sections, except those of *P. qugwadi* infecting *P. yessoensis* (Table 1 and Fig. 4A–1). No hybridization to parasite cells of other genera was observed. ISH of parasite cells in tissue sections of infected Korean Manila clams with this genus-Perkinsus probe confirmed the genus level affiliation of the parasites in our sample of juvenile Korean Manila clams (Fig. 5).

**DISCUSSION**

We report the confirmation by ISH assays and histology of Perkinsus sp. infections in Manila clam seed proposed for the introduction into Mexican waters and the subsequent transport to growout sites on the Pacific coast of the United States. This is the first confirmation by a molecular diagnostic probe of Perkinsus sp. infection of Korean Manila clams. As a result of these findings, the plan for importation of these clams was rejected by the shellfish producer, and no Korean seed clams were imported to the west coasts of Mexico or the United States. However, the ready avail-

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**Figure 3. Cyst of Perkinsus sp. trophozoites encapsulated by a hemocyte within the gill of a Manila clam. Bar, 10 µm, H&E.**

**Figure 4. Tissues sections of host tissues reacted with the genus-Perkinsus probe Perksp700 by ISH. Positively stained Perkinsus sp. parasites are shown by arrows.** (A) *P. marinus* in *C. virginica* intestine (bar, 10 µm). (B) Perkinsus sp. in *C. pacifica* (bar, 10 µm). (C) *P. atlanticus* in *R. decussatus* (bar, 10 µm). (D) *P. olseni* in *H. laevigata* gill and mantle (bar, 25 µm). (E) Perkinsus sp. in *M. bathica* (bar 25 µm). (F) Perkinsus sp. in Japanese *V. philippinarum* (bar, 10 µm). (G) *P. chesapeakt* in *M. arenaria* (bar 10 µm). (H) *P. mediterraneus* n. sp. in *O. edulis* (bar, 10 µm). (I) *P. qugwadi* in *P. yessoensis* (no hybridization observed) (bar, 25 µm).
ability of such infected seed clams from Korean or Japanese producers requires vigilance to ensure that no such importations take place into areas that are free of the pathogen, such as the west coasts of North and Central America. Reports of lethal Perkinsus sp. infections in European and eastern Asian Manila clams from latitudes as far north as that of northern Oregon, confirm the high likelihood that such infections, if introduced, could persist and be transmitted, with damaging results to both wild and cultured clam stocks along the Pacific coasts of North and Central America.

This study demonstrated that infection prevalence in seed clams ranging from 16 to 32 mm SL can be nearly 100% and that high parasite intensities cause significant histologic damage to the organs of infected clams, particularly the gills.

Choi and Park (1997) studied five species of Korean clams for infections by Perkinsus sp. using Ray's fluid thioglycollate medium (Ray 1966) and found infected Manila clams along the south coast of Korea. While no infection occurred in clams of <15 mm SL, nearly 100% infection prevalence occurred in clams of >20 mm SL. Park et al. (1999) reported mass mortality of Manila clams along the west and south coasts of Korea over a period of several years, which was associated with Perkinsus sp. infections. They reported 100% infection prevalence in 142 clams from Komsee Bay on the west coast of Korea with moderately severe mean parasite intensities of 2.87 based on the infection intensity scale of Choi et al. (1989). A negative correlation was found between the intensity of Perkinsus sp. infections and the clam condition index, while clam size was positively correlated with infection intensity.

Maeno et al. (1999) reported Perkinsus sp. parasites in Manila clams from an inner bay of the western part of Japan in April 1998, using genus-Perkinsus-specific antibodies. These authors concluded that the parasites were Perkinsus sp. based on a positive reaction with both single and clustered trophozoites. Hamaguchi et al. (1998) have reported the first detection of Perkinsus sp. in Japanese Manila clams. Anecdotal information that we received from the Korean supplier of the seed clams and their Japanese customers indicated that the Manila clam seed had been transported from the Korean source to Japan for at least 20 y with no unusual mortalities or loss of growth reported. This anecdotal report and the multiple reports of the Perkinsus sp. parasite occurring about 1997 or 1998 in Japan and Korea suggest that it could have been a new introduction to the Korean clams, as well as the Japanese clams, at about this time.

Manila clams and other bivalve species from Europe reportedly have been infected with Perkinsus sp., as follows: P. atlanticus from the Mediterranean coast of Spain (region of the Ebro Delta, Tarragona, Spain) infected R. philippinarum (Sagrista et al. 1996); Manila clams from the Lagoon of Venice in northeast Italy infected with a Perkinsus sp. (DaRosa et al. 1998); and P. atlanticus infected the carpet shell clam (R. decussatus) from European locations (Ordas et al. 2000). Villalba et al. (2000) reported a significant correlation between the SL of R. decussatus and P. atlanticus infection intensity. No clams of <20 mm SL were infected, and the highest seasonal parasite intensities occurred in spring and late summer to early autumn.

The relationship of Perkinsus sp. in European waters to the Perkinsus sp. found in Korea and Japan is unknown at this time. Nonetheless, this and other studies cited in this report indicate the presence of this damaging parasite in Korean and Japanese Manila clams, confirmed first in this study by histology and then definitively by the Perkinsus sp.-specific probe presented for the first time in this article. This knowledge can be used to prevent the unintentional introduction of this parasite to west coast of North and Central America. We urge that the science presented in this article be applied by shellfish growers, and by natural resource and conservation managers to prevent such a damaging introduction.

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