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NATURAL AND EXPERIMENTAL INFECTION OF *MERCENARIA MERCENARIA* BY *PERKINSUS MARINUS* FROM *CRASSOSTREA VIRGINICA*

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ABSTRACT: Histological examination of wild northern quahog clams, *Mercenaria mercenaria*, from Warsaw Flats and vicinity on the Morgan River, South Carolina, USA, revealed that some harboured a *Perkinsus*-like protist. Based on the hypothesis that the parasite in clams may represent *P. marinus* from oysters, *Crassostrea virginica*, experimental infections were carried out by exposing uninfected clams raised in controlled hatchery facilities to wild oysters from areas where the prevalences of *P. marinus* were known to be high. After 10 days of exposure to oysters (experimentals) or to no oysters (controls), half of the clams were examined histologically while the remaining clams were subjected to modified "panning". It was ascertained that *P. marinus* can be transmitted from infected oysters to clams. The modified panning technique was demonstrated to be comparable in accuracy as histology in diagnosing *P. marinus* infections.

KEY WORDS: Clams, *Mercenaria mercenaria*, oysters, *Crassostrea virginica, Perkinsus marinus*, protistan pathogen, parasite.

INTRODUCTION

During June, 1992, four specimens of the northern quahog clam, *Mercenaria mercenaria*, collected at Warsaw Flats on the Morgan River, Beaufort County, South Carolina, USA, were prepared for histological examination. Three of these clams had chalky, pitted, grayish-brown shells. All four specimens measured between 30 and 40 mm in length. Examination of 7-µm sections stained with hematoxylin and eosin revealed that the three clams with abnormal shells were infected with a *Perkinsus*-like protist that was morphologically indistinguishable from *P. marinus.*

Subsequent anecdotal information obtained by one of us (VGB) from shellfishermen in the Morgan River area during the summer of 1993 suggested that there had been mass mortalities of *M. mercenaria* during the previous four years. At one time a small clamming industry existed in this area. Field studies have confirmed that *M. mercenaria* were indeed sparse in the Morgan River and at Warsaw Flats. This led us to investigate the possible cause(s) of the significant depletion of the clam population.

MATERIAL AND METHODS

Wild clams: On June 16, 1993, five specimens of *M. mercenaria* were collected from an intertidal oyster bar in the Morgan River near the Dawtaw Marina, Beaufort County, South Carolina, USA. The salinity at the site was 27‰ and the surface water temperature was 28° C. An additional four clams were collected at Warsaw Flats in the same drainage on June 18, 1993, after an 8-hour search. The salinity at this site was 24‰ and the surface water temperature was 29° C. All of the wild clams measured between 5 and 7,5 cm long.

Experimental clams: All of the clams employed in the experimental infection studies were raised from larvae in controlled hatchery facilities at the SeaPerfect's Atlantic LittleNeck ClamFarms, James Island, South Carolina. Continuous examination of representative clams being raised in these facilities had revealed the absence of protistan parasites. Clams from this source employed in the experimental infection studies and comprising the control group measured 5-7 mm long.

Infected oysters: It has been known for many years that a high percentage of the eastern oyster, *Crassostrea virginica*, along the South Carolina, USA' coast is infected with *P. marinus* (BURRELL, 1986). Although continuous surveillance has revealed that the infection incidence may be as high as 100% in some areas, mass mortalities usually only occur during periods of elevated temperatures and severe drougtht (CHENG & COMBES,1990).

As a part of this continuous survey of *C. virginica* in South Carolina, it has been determined that 62% of oysters (n = 82) growing in Church Creek, Johns Island (average salinity 26,5‰), collected during July, 1993, harboured *P. marinus*. It was also ascertained that 70% of the oysters (n = 50) growing in Charleston Harbor (average salinity 30,4‰) collected during August, 1993, harboured *P. marinus*.

Experimental exposures: To determine if *P. marinus* from naturally infected *C. virginica* can invade *M. mercenaria*, two series of bivalve exposures were established. Comprising the first, designated as Ser. A, were 18, 400-ml beakers each containing 250 ml of filtered seawater (26,5‰ salinity). One oyster (4,0-4,4 cm long)

collected from Church Creek and two laboratory raised, uninfected clams were placed in each beaker. The second series, designated as Ser. B, consisted of 18, 400-ml beakers containing 200 ml of filtered seawater (30,2‰ salinity), two oysters (3,0-3,6 cm long) collected from Charleston Harbor, and three laboratory raised, uninfected clams.

The control series of the exposure experiment consisted of 10, 400-ml beakers each containing 250 ml of filtered seawater and three laboratory raised, uninfected clams. All of the beakers comprising Ser. A and B and the control series were maintained at 28-30° C for 10 days. These relatively high temperatures were employed because observations over the years on *P. marinus* infection in *C. virginica* suggested that the intensity and prevalence of infection are highest during the hottest summer months.

Examination for possible infections: At the termination of the 10-day exposure period, the shells of nine of the oysters and 18 clams from the same nine beakers (Ser. A) were removed and the soft tissues were fixed individually in 10% seawater-formalin. Similarly, the soft tissues of 18 oysters and 27 clams from the same nine beakers (Ser. B) were fixed in seawater-formalin. All of the fixed bivalves were subsequently processed for histological study. Seventy 7-µm sections of each bivalve, stained with hematoxylin and eosin (H & E), were examined. In addition to oysters and clams from Ser. A and B that were processed for histological sudy, 20 sections from each of 15 clams from five beakers of the control series were similarly processed for histological examination.

Coincidental to histological examination, 1-ml hemolymph samples were collected from the adductor muscle sinus of each of the nine remaining oysters of Ser. A and each of the remaining 18 oysters of Ser. B. Also, 0,5-ml hemolymph samples were collected from the posterior adductor muscle sinus of each of the remaining 18 clams of Ser. A and the remaining clams of Ser. B. Each of the hemolymph samples were subjected to a modification of the panning technique for the identification of protistan parasites (FORD *et al.*, 1990). This technique, originally developed to concentrate the plasmodial stages of another protistan parasite, *Haplosporidium nelsoni*, in oyster hemolymph, has been found to be a reliable method for determining the presence or absence of both *H. nelsoni* and *P. marinus* in oyster hemolymph (CHENG & DOUGHERTY, 1994). In brief, the hemolymph samples were carefully placed on the bottom of a Petri dish with a Pasteur pipette and permitted to settle for 30 min. As the meronts of *P. marinus* and the plasmodia of *H. nelsoni* generally are not as adherent to glass as bivalve hemocytes, a wet smear of the supernatant will readily reveal the presence or absence of parasites. In addition to the hemolymph samples from the bivalves from Ser. A and B, similar samples were collected from the 15 clams of the control series. These were also subjected to panning.

RESULTS

Histological findings

Among the nine oysters from Ser. A that were examined histologically, six were determined to be infected with *P. marinus*. Among the 18 clams from the same series, 10 were infected with *P. marinus*. Moreover, it was determined that in each instance where the oyster was uninfected, the two clams that shared the same beaker were uninfected. Also, only in two instances where the oyster was infected was only one of the clams infected.

Among the 18 oysters from Ser. B that were examined histologically, 12 were infected with *P. marinus*. Among the 27 clams from Ser. B, 16 were infected. It is noted that there was only one instance where all three clams from the same beaker were found to harbour *P. marinus* and in this case, both of the oysters in the same container were heavily infected. In three additional beakers where both oysters harboured *P. marinus*, two of the three clams became infected. Also, in three of the beakers where only one oyster was infected, two of the three clams were infected while in an additional beaker that contained a single infected oyster, only one clam became infected. In the one beaker that contained two uninfected oysters, none ot the three clams became infected. None of the clams of the control series that were examined histologically harboured *P. marinus*.

In each case where any oyster or a clam harboured *P. marinus*, the two life-cycle stages most readily recognized were mature meronts (= trophozoites), each measuring 12-18 µm, enclosing a conspicuous vacuole, and large mother meronts.(mother cells), each measuring 13-20 µm, enclosing eigth or more daughter meronts. These intramolluscan life-cycle stages were situated primarily in connective tissue peripheral to the digestive tract, although some parasite-caused lesions also occurred in the ctenidial matrix, gonads, and between myofibers of the foot. Each lesion was comprised of host hemocytes surrounding clusters of protists. Some of the parasites had been phagocytosed by clam granulocytes.

Panning results

Of the nine oysters from Ser. A hemolymph samples that had been subjected to panning, six were infected with *P. marinus*. Among the 18 clams from Ser. A, eigth were determined to harbour *P. marinus*. Of the 18 oysters from Ser. B, 14 were determined to be infected with *P. marinus* by panning. Of the 27 clams from Ser. B, 15 were infected.

It is noted that among the oysters and clams placed in nine beakers of Ser. B designated for panning, three of the clams were infected in only three containers in which both oysters were infected with *P. marinus*. In an additional single beaker in which both oysters were infected with *P. marinus*, two of the associated clams were infected. In another three beakers in which both oysters in each were infected, only one of the clams was infected. In two additional beakers where only one of the two oysters in each was infected, only one of the three associated clams were infected. Finally, in the last beaker in which neither oysters where determined to be infected with *P. marinus*, all three associated clams were also not infected.

All of the 15 clams on the control series that had been subjected to panning were determined not to be uninfected.

DISCUSSION

The earliest report that *P. marinus* may be infective to *M. mercenaria* is that by ANDREWS (1954) who reported

that during 1953-54, a *P. marinus*-like parasite (designated as *Dermocystidium marinum* as it was considered to be a fungus) was found in samples of *M. mercenaria* collected near Gloucester Point, Virginia, USA. In addition to *M. mercenaria*, *P. marinus* or species of the same genus were reported from 11 other species of bivalves from Virginia's tidal waters. More recently, PERKINS (unpubl.) found that 4,26% of *M. mercenaria* from the York River in Virginia, collected in October, 1991, and 10% of the same clams from Wachapreague, Virginia, collected in June, 1992, to be naturally infected with *Perkinsus* sp. The fluid thioglycolate medium (FTM) technique (RAY, 1954a) was employed by both ANDREWS (1954) and PERKINS (unpubl.) to detect the parasites.

Although experimental transmission of *P. marinus* from oyster (*C. virginica*) to oyster has been successfully accomplished (MACKIN, 1952; MACKIN, RAY & BOSELL, 1953; RAY, 1954a), its transmission from *C. virginica* to other bivalve species has not been reported in the literature. RAY (1954b) and ANDREWS & HEWATT (1957) reported that they were unable to induce invasive infections of *P. marinus* in *M. mercenaria* using the techniques of feeding and injecting minced, infected oyster tissues. RAY (1954a), however, was able to detect the presence of *P. marinus* in tissues of *M. mercenaria*, using the FTM technique but only after the mince was injected between the shell and mantle, not when the mince was fed to clams. Those parasitic cells detected were only found near the site of injection. Based on these results, RAY (1954a) concluded that rigid host specificity of *P. marinus* exists.

The concept of narrow host specificity of *Perkinsus* spp. has since been challenged by the results of GOGGIN, SEWELL & LESTER (1989) who were able to experimentally cross-transmit several *Perkinsus* spp. from Australian bivalves and gastropods into nine other mollusc species by exposing them to zoospores generated from pre-zoosporangia held in Petri dishes containing sea water.

In view of the field observations by ANDREWS (1954), PERKINS (1985), and GOGGIN & LESTER (1987), the experimental results of GOGGIN, SEWELL & LESTER (1989) as well as those reported herein, it would appear that *Perkinsus* spp. are infective for a wide range of hosts. Furthermore, *P. marinus* is infective for more species of bivalves than just *C. virginica*.

It is noted that FONG *et al.* (1993), by employing the polymerase chain reaction and molecular cloning, have reported that the small subunit rRNA gene of *P. marinus* consists of 1793 nucleotides and the sequence has 97,5% similarity to that of *Perkinsus* sp. from the Australian bivalve *Anadara trapezia.* Also, GOGGIN (1994) by comparing the internal transcribed spacers (ITS) and 5.8S regions of the rRNA gene from five isolates of *Perkinsus* from all over the world (*P. marinus* from American *C. virginica, P. atlanticus* from Portuguese *Ruditapes decussatus, P. olseni* from *Haliotis laevigata, Perkinsus* sp. from *Chama pacificus*, and *Perkinsus* sp. from *Anadara trapezia*; the latter three all from Australia) has reported that the lengths and sequences of ITS1 and ITS2 of *P. marinus* differ significantly from those of the four isolates from Portuguese and Australian molluscs. There were also differences between the sequences of the ITS2 region of the four isolates other than *P. marinus.* It remains unknown whether these variations reflect differences in host specificity ranges among *Perkinsus* spp.

It is also noted that the use of minced, infected oysters, whether by feeding (RAY, 1954a; ANDREWS & HEWATT, 1957) or by injection (RAY, 1954a) did not yield sustained infections in *M. mercenaria* even though the cells of *P. marinus* (i.e., meronts, merozoites, schizonts), which would initiate infection in *C. virginica*, were present. As GOGGIN, SEWELL & LESTER (1989) were successful in demonstrating low host specificity when they used zoospores as the infective cells, the possibility exists that these flagellated cells are required to initiate infections when crossing host generic or species lines. Also, it is possible the threshold numbers of cells required for initiation of infection are higher when nonflagellated cells are used. PERKINS (unpubl.) has found that zoospores of *Perkinsus atlanticus* from *Macoma balthica* readily cause noninvasive infections in *C. virginica* but was unable to initiate infections using meronts, merozoites, and schizonts in minced *M. balthica* tissues.

Based on our findings involving both histological examination and panning, of the 18 oysters collected from Church Creek, 12 (66,67%) were naturally infected with *P. marinus* and among the 36 clams that had been exposed to oysters from this site, 18 (50%) had become infected. Similarly, among the 36 oysters originating from Charleston Harbor, 26 (72,22%) were naturally infected and among the 54 clams that had been experimentally exposed to the Charleston Harbor oysters, 31 (57,41%) had become infected with *P. marinus.*

A comparison of the results obtained by the panning technique with those obtained by histological examination in the South Carolina study shows that in the case of oysters exposed from Church Creek (Ser. A) and Charleston Harbor (Ser. B), the former method revealed 77,78% infection while the latter revealed 66,67% infection. In the case of clams exposed to Church Creek oysters (Ser. A), panning revealed 44,44% infection while histological examination revealed 55,56% infection. Also, panning revealed 55,56% infection with *P. marinus* in clams exposed to Charleston Harbor oysters while histological examination indicated 59,26% infection in those exposed to Charleston Harbor oysters (Ser. B). These results indicate that the modified panning technique employed by us is a reliable, qualitative diagnostic method.

The pathogenicity of *P. marinus* to *C. virginica* is well known (MACKIN, OWEN & COLLIER, 1950; MACKIN, 1953, 1961; RAY, 1954a, b; KERN, SULLIVAN & TAKATA, 1973; ANDREWS, 1979; and numerous others).

The pathogenicity and lethality of *P. marinus* to *M. mercenaria*, however, remains uncertain. As stated, one of us (VGB) has received anecdotal information from shellfishermen in the Morgan River area that there have been mass mortalities of wild *M. mercenaria* during 1988-1992. It is noted that DA ROS & CANZONIER (1985) attributed mass mortalities of *Ruditapes decussatus* and *R. aureus* from the Italian coast to the *Perkinsus* sp. found by them. Also, although a causal relationship has not been established, FIGUERAS, ROBLEDO & NOVOA (1992) reported a *Perkinsus*-like organism and an unknown haplosporidian associated with abnormally high mortality of carpet-shell clams, *R. decussatus*, imported to Meira, Spain, from Portugal.

There are earlier reports of other species of clams being infected with *Perkinsus* spp.. In addition to those found along the west and south coasts of Australia (GOGGIN & LESTER, 1987), AZEVEDO (1989) described *P. atlanticus* which causes motalities of *Ruditapes decussatus* in Portuguese waters. DA ROS & CANZONIER (1985) and GOGGIN (1992) reported *Perkinsus* spp. in diverse bivalves from the Mediterranean Sea and Atlantic coast of France, including the clams *R. decussatus, Tapes phillipinarum, Venus aureus*, and *V. verrucosa.* GOGGIN (1992), however, did not find *Perkinsus* sp. in 10 *M. mercenaria* from the west coast of France. Along the east coast of the United States, *Perkinsus* sp. has been known for some time to be a parasite of *Macoma* spp. (RAY, 1954a).

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