

A Specific DNA Probe to Identify the Intermediate Host of a Common Microsporidian Parasite of *Penaeus merguensis* and *P. monodon*

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Abstract

The intermediate host required for transmission of a common microsporidian (*Agmasoma*) parasite of the penaeid shrimp, *Penaeus merguensis* and *P. monodon* is still unknown, so cross-infection studies and studies to develop preventive or therapeutic measures are not possible. The purpose of this study was to develop a specific nucleic acid probe to aid in finding the intermediate host, and in tracing the life cycle of the parasite. To prepare the probe, DNA was extracted from purified microsporidian spores derived from infections in *P. merguensis* and *P. monodon*. The prepared genomic DNAs were then incompletely digested with *Sau3AI* and fragments were ligated to the pGEM7Zf+ vector before being transformed to *Escherichia coli* JM109. Candidate probes were screened by *in situ* colony hybridization, slot blot hybridization and Southern blot hybridization using dATP^{32P} labelled *Agmasoma* DNA. All the candidate probes from each *Agmasoma* source cross-hybridized with whole *Agmasoma* DNA digest from the other source and with roughly the same quality of signal. Finally, a probe (1-mo) derived from *Agmasoma* of *P. monodon* gave the best sensitivity because it was found to be derived from a multicopy sequence and it did not cross-hybridize with other organisms from shrimp ponds (i.e., both shrimp species, *Artemia*, mixed protozoa, *E. coli* and *Vibrio parahaemolyticus*). DNA extracts were prepared from 22 animal species (including fish, crustaceans, molluscs and mixed plankton) present in seawater canals where microsporidian-infected shrimp were present. These were probed with digoxigenin (DIG) labelled 1-mo probe. Two fish species, *Priacanthus tayenus* and *Scatophagus argus*, gave positive hybridization signals.

Introduction

Microsporidia are obligate intracellular parasites which can infect all animal phyla, both vertebrate or invertebrate, including other protozoans such as ciliates, myxosporidia and gregarines (Sprague 1977). Microsporidia lack mitochondria and their ribosomes have been reported to be 70s, similar in size to those in prokaryotes (Vossbrinck and Woese 1986; Vossbrinck et al. 1987). On the other hand, they do have nuclear membranes, the definitive characteristic of eukaryotic cells (Vavra 1976; Cavalier-Smith 1987).

The penaeid shrimp, *Penaeus merguensis* and *P. monodon*, are important products in the worldwide seafood business, and they sometimes develop microsporidian infections that result in economic loss to shrimp farmers. The disease causes an opaque white coloration in normally transparent tissue when it has been replaced by microsporidian spores. This seems to occur without invoking a major host inflammatory response. The condition is called "white back" in Thai (Flegel et al. 1992) and "milk shrimp" or "cotton shrimp" in English (Lightner 1988). The discolored shrimp fetch a very low market price. Besides the "white back" symptom, the parasite affects other internal organs such as the hepatopancreas and the gonads. According to Lightner (1988), "infected shrimp exhibit poor stress resistance and have poor stamina. Thus, they may be prone to loss from predation and to poor survival following handling. Infection of gonads causes sterility and may cause feminization of infected male shrimp." Lightner also states that "infection seems to inhibit normal migration in wild shrimp."

Many microsporidian species have specific hosts and the life cycles for most are still unclear (Canning and Hollister 1987, 1991), even though they have been studied in some detail, especially for groups such as the arthropods. For example, the life cycles of microsporidians in penaeid shrimp are still unclear although they have been known for many years. By contrast the life cycle of *Amblyospora californica*, a microsporidian parasite of mosquitoes, has been thoroughly explained and shown to be somewhat complex (Becnel 1992). It involves two alternate hosts with three spore stages and both vertical and horizontal transmission.

In Thailand, the main cultivated shrimp species are *P. merguensis* and *P. monodon* (Donyadol et al. 1985). Both of these species are infected by what appears to be a single microsporidian parasite, based on morphology with the light and electron microscopes (Flegel et al. 1992). This parasite has been tentatively identified as *Agmasoma penaei* (Hazard and Oldacre 1975; Flegel et al. 1992). However, cross-infection studies would be required to conclusively prove that this microsporidian is capable of infecting two species of shrimp. These cannot be carried out since the intermediate host remains unknown. The classical method to study microsporidian life cycles is to use the techniques of bioassay combined with microscopy. This often requires a reasonable amount of good fortune to succeed, especially in cases where the number of candidate intermediate hosts is large. This is the general situation with penaeid shrimp. The process is also complicated by the fact that the morphology of microsporidia can vary with the host.

The aim of this study was to develop a specific molecular genetic probe which would allow for easy and specific screening of candidate intermediate hosts. Such a probe would make it possible to screen a large number of candidate hosts without microscopic examination. The probe could also be used to trace the fate of the parasite under various conditions, and to help determine whether transmission is possible by the transovarial route, even if parasite morphology could not be resolved.

Materials and Methods

Specimens

Specimens of *P. merguensis* and *P. monodon* with visibly whitened tissues (i.e., cotton shrimp or white back shrimp) were collected from shrimp ponds and seawater supply canals in Songkhla province in southern Thailand in June-July 1991. Infection by *Agmasoma* was confirmed by microscopic examination of wet mount preparations of whitened tissues for the presence of pansporoblastic vesicles containing eight ovoid spores (Flegel et al. 1992). Specimens were frozen and transported to Bangkok where they were stored frozen (-20°C) until processed for preparation of spores. A pure culture of *Vibrio parahaemolyticus* from an infected farm specimen of *P. monodon* was obtained from the microbiology laboratory of Aquastar Laboratories Ltd., while *Escherichia coli* consisted of the host transformation bacteria JM109 and DH5 α . Other organisms used for DNA hybridization tests were obtained from water supply canals to shrimp farms in Songkhla. Also in Songkhla at the farm of Aquastar Co. Ltd., samples of the diatom *Skeletonema*, and the brine shrimp *Artemia* were obtained from larval feed cultivation tanks, and mixed protozoa were obtained by recovering floating exuvia of *P. monodon* postlarvae from nursery tanks. DNA from these whole organism samples (or guts only for the fish) was extracted directly from the freshly collected material using the same method described below for DNA extraction from *Agmasoma* spores. Since they came from outdoor, non-sterile sources, these specimens provided mixed DNA extracts.

Preparation of Agmasoma Spores

Agmasoma spores from thawed, infected shrimp tissues were prepared by tissue grinder homogenization in sterile distilled water followed by centrifugation at 3,000 rpm for 10 minutes (Kubota centrifuge model KR20000T, rotor RA3A). After resuspension and counting with a hemacytometer, the suspension was diluted with sterile distilled water to a concentration of $3\text{-}5 \times 10^7$ spores $\cdot\text{ml}^{-1}$. This suspension was layered onto the top of a discontinuous 20% plus 40% sucrose gradient solution. After centrifugation at 2,000 rpm for 10 minutes (Kokusan centrifuge model H103, rotor RF110), most of the spores were located at the interface between the 20% and 40% phases. This spore fraction was collected and washed with distilled water two to three times by centrifugation at 4,000 rpm for 10 minutes. The resulting pellet was then

resuspended and repurified by a second separation on a 20% plus 40% discontinuous sucrose gradient followed by washing with distilled water until all of the sugar was removed (i.e., four to five times). The purity of the microsporidian spore preparation was checked by the light microscope using Gram's stain. This preparation was referred to as "purified spores."

Preparation of Agmasoma DNA

Purified *Agmasoma* spores were suspended in sterile triple-distilled water at a concentration of 5×10^6 spores·ml⁻¹. The DNA was extracted after Sambrook et al. (1989) with slight modification. Specifically, an equal amount of lysis buffer (50 mM tris-HCl, pH 9.0, 100mM EDTA, 50 mM NaCl and 2% SDS) supplemented with 0.5 µg·ml⁻¹ of proteinase K was added to the spore suspension. After incubation at 55°C for 2-12 hours, the digested sample was treated with phenol and chloroform and the DNA was precipitated by addition of absolute ethanol. The pellet was then washed twice with 70% ethanol. The DNA was dried in a vacuum dessicator and resuspended in sterile triple-distilled water. The quantity of DNA was measured either by spectrophotometer using the wavelength ratio of 260 nm: 280 nm, or by agarose gel electrophoresis, staining with ethidium bromide and visualization with a UV transilluminator (Sambrook et al. 1989).

Construction and Screening of Agmasoma Probes

The techniques used for construction of nucleotide probes were based mainly on the techniques of Sambrook et al. (1989) with slight modification. Specifically, the *Agmasoma* DNA was partially cleaved with Sau3AI enzyme at 37°C for 15 minutes at a ratio of 1 µg DNA to 4 units of enzyme. The digestion reaction was stopped by heating at 65°C for 10 minutes. The Sau-3AI digested DNA was then ligated to dephosphorylated BamHI pGEM7Zf+ at a weight ratio of 5:1 (DNA:plasmid) at 14°C for 20 hours and transformed to an *E.coli* host JM 109 (Yanish-Perron et al. 1985). The transformed *E. coli* JM 109 were then spread on Xga/IPTG ampicillin plates. To form the probe library, 200 white and ampicillin-resistant colonies containing presumptive recombinant plasmids were randomly selected for the DNA from each *Agmasoma* source. These colonies were subcultured on new ampicillin plates which were kept as master plates for selection of potential *Agmasoma*-specific clones by the colony hybridization technique using dATP³²P labelled *Agmasoma* DNA and dATP³²P labelled shrimp DNA (both shrimp species) as the probes. Colonies reacting to the *Agmasoma* probe but not to the shrimp probes (positive clones) were selected for further processing. After growth in LB-broth, recombinant plasmids were extracted from the positive clones using a rapid lysis protocol. Slot blot hybridization and Southern blot hybridization were used to verify that the recombinant plasmids hybridized strongly with *Agmasoma* DNA but did not hybridize to shrimp DNA. For Southern blot hybridizations, recombinant plasmids were digested with EcoRI/BamHI, electrophoresed and transferred to nitrocellulose filters, followed by hybridization with dATP³²P labelled shrimp (two species) and *Agmasoma*

DNA. Clones passing this test were used for further specificity and sensitivity tests.

Specificity and Sensitivity Tests

Candidate probes were checked for specificity by hybridization tests using DNA extracted from several live organisms found normally around shrimp ponds. These included mixed protozoa in shrimp exuvia, diatom larval feed (*Skeletonema* sp.), brine shrimp (*Artemia*), mixed crustaceans from pond plankton (copepods, cladocerans and planktonic shrimp), *E. coli* and *V. parahaemolyticus*. The sensitivity of candidate probes was studied by diluting the microsporidian DNA in DNA diluent buffer (Boeringer) followed by application to pretreated nitrocellulose filters, denaturation with the 0.5 M NaOH/1.5 M NaCl for 5 minutes (two times) and neutralization with 0.5 M Tris-HCl pH7.5/1.5 M NaCl for 5 minutes (two times). In addition, the probes were labelled with dATP³²P and hybridized to Southern filters containing Sau3AI partially digested *Agmasoma* DNA, as well as penaeid shrimp DNA (both species).

Labelling and Hybridization Conditions

Labelling of microsporidian genomic DNA and various plasmids was carried out by either of two main methods. The first method consisted of labelling with dATP³²P using a random primer labelling kit (Amersham) and detection using X-ray film exposure. The second method consisted of digoxigenin labelling (DIG labelling) using a commercial kit (Boeringer) and detection with ELIZA according to the Boeringer manual. For DNA hybridization using radioisotope labelling, samples were processed for 15 hours at 60°C using 2-3 x 10⁶ cpm per ml of hybridization solution. For DIG labelling, 30 ng of DIG labelled probe was used per ml of hybridization solution. The hybridized filters were washed twice for 15 minutes under highly stringent conditions (0.1 x SSC, 1% SDS) at 70°C before detection of hybridization signals.

Sequencing and Characterization of Probes

A single probe (1-mo) with the highest specificity and sensitivity among the candidate probes was selected for further characterization. This probe was subcloned by digestion with EcoRI, and a fragment of approximately 3.8 kb was selected and religated using low melting agarose gel. After amplification in *E. coli* grown in ampicillin-containing LB-broth, the plasmid containing the subcloned fragment was purified by the polyethelene glycol technique. Finally, single stranded plasmid prepared according to Messing et al. (1977) was sequenced using "-21M13" as the forward primer for upstream sequencing. For reverse sequencing, the purified double stranded plasmid was used with the M13 reverse primer (M13RP1). For both sequencing operations a tag dye primer sequencing kit was used with an automatic sequencing analyzer (Applied Biosystems, model 373A), and analyzed with the DNA-SIS program using a personal MS-DOS computer.

Results

Isolation of Agmasoma Spores

The yield of *Agmasoma* spores from white back shrimp depended upon the severity of infection, with severe infections giving the highest yields. Usually, purified spores could be obtained after only two separations using sucrose gradients. In some cases, however, further separation was needed if microscopic examination of the preparation showed some contaminating material. The purity of the preparations was determined by comparison with unpurified samples using light microscopy (100 x objective) with Gram's stain preparations as shown in Fig. 1. By this method, there was no indication of any contamination by the host shrimp or by other organisms such as bacteria. The estimated recovery of spores was 50-65%.

Preparation of Agmasoma DNA and Probe Construction

Agmasoma genomic DNA was extracted by slight modification of the method of Sambrook et al. (1989) in that the concentration of proteinase-K was increased to 0.5 μg per ml of the lysis buffer. A lesser amount of proteinase-K

sometimes resulted in DNA degradation. With 0.5 μg per ml of the lysis buffer, the microsporidian genomic DNA always came out as an intact band when electrophoresed in 0.7% agarose gel.

Ligation of the partially digested *Agmasoma* DNA to pGEM7Zf+ and transformation of *E. coli* resulted in 7-12 white, ampicillin-resistant colonies per ng of genomic DNA used in the ligation reaction. However, it was later found that not all these clones possessed inserted fragments. After selection by *in situ* colony hybridization, slot blot hybridization and Southern blot hybridization, the candidate probes 1-me derived from *Agmasoma* of *P. merguensis*,

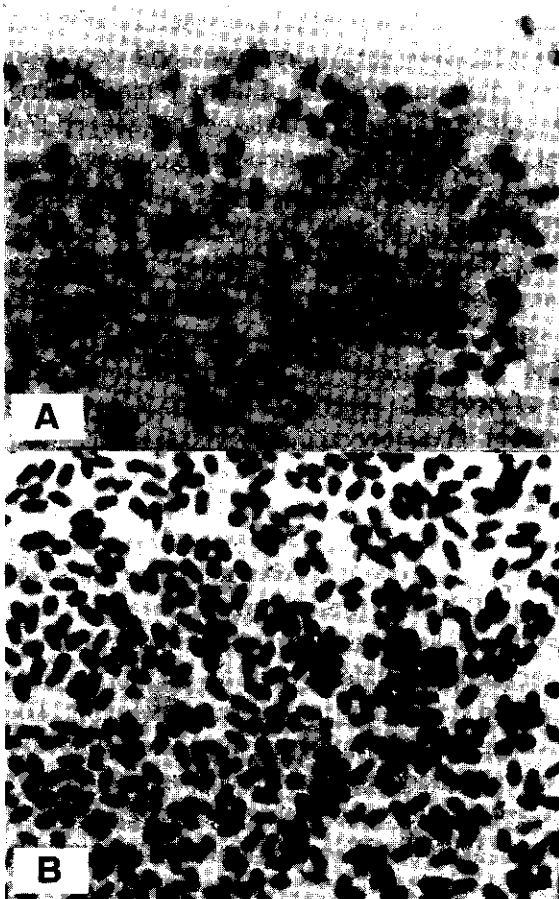


Fig. 1. Photomicrographs of unpurified (1A) and purified (1B) *Agmasoma* spores

and 1-mo derived from *Agmasoma* of *P. monodon* were selected for further study.

Specificity and Sensitivity

The sensitivity of 1-me was 1 ng for *Agmasoma* DNA from both host sources (Fig. 2), while the sensitivity for 1-mo was 0.01 ng for both (Fig. 3). The probes gave specific signals only to *Agmasoma* DNA, and no signal expression, even at DNA concentrations of 200 ng, to DNA from both host shrimp, *Artemia*, mixed protozoa, mixed plankton, *E. coli* and *V. parahaemolyticus*. Southern blots using dATP³²P labelled 1-me and 1-mo to hybridize with Sau3AI partially digested *Agmasoma* DNA from both shrimp species gave identical hybridization patterns (Fig. 4 and 5, respectively). For probe 1-me, a multiband hybridization pattern was obtained which was the same whether using inserted DNA fragments or the whole recombinant plasmid. There was no signal with similarly prepared DNA from either of the host shrimp species (Fig. 5) or from *E. coli* (data not shown). Probe 1-mo was later subcloned from approximately 2.6 kbs to 736 base pairs (1.1-mo). Probe 1.1-mo had essentially the same specificity and sensitivity as 1-mo (data not shown).

Characterization and Sequencing of the Probe

The probe 1.1-mo derived from the probe 1-mo was sequenced and shown to be composed of mainly A and T bases (66% of the 736 base pairs). The sequence of the probe and the restriction map are being withheld for proprietary reasons. The sequence showed no homology to any known sequence in the GenBank database.

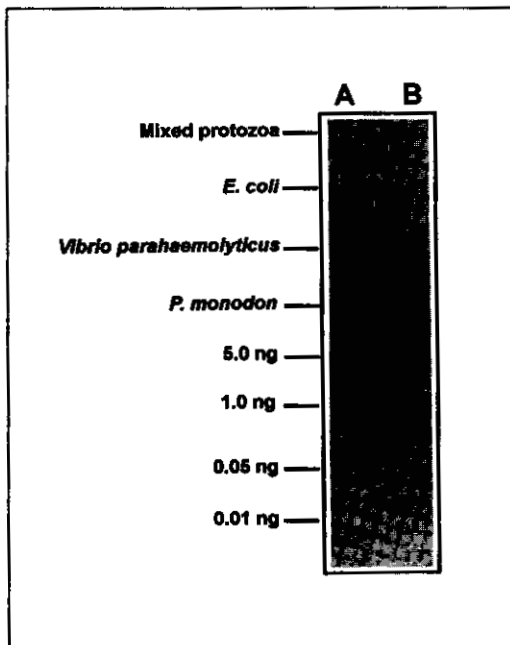


Fig. 2. The sensitivity of probe 1-me. *Agmasoma* DNA from *P. merguensis* (A) and from *P. monodon* (B) was blotted onto the filter (nanogram quantities indicated to the left of the filter) along with DNA (200 ng) from other organisms indicated. The filter was then probed with DIG labelled 1-me. The sensitivity was 1 ng of *Agmasoma* DNA from either source.

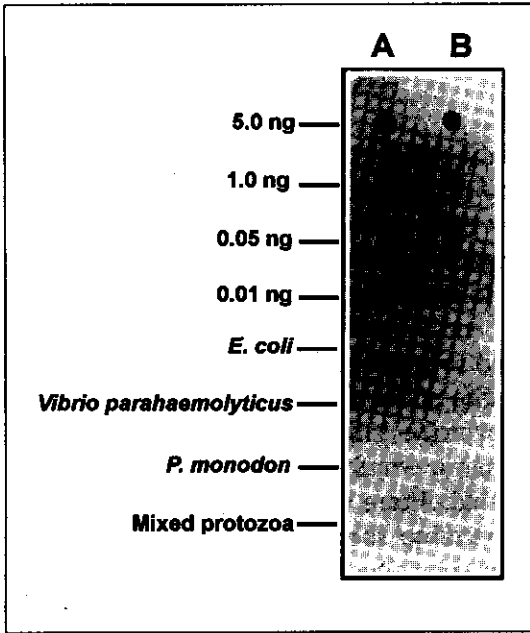


Fig. 3. The sensitivity of probe 1-mo. *Agmasoma* DNA from *P. merguensis* (A) and from *P. monodon* (B) was blotted onto the filter (nanogram quantities indicated to the left of the filter) along with DNA (200 ng) from other organisms indicated. The filter was then probed with DIG labelled 1-mo. The sensitivity was 0.01 ng of *Agmasoma* DNA from either source.

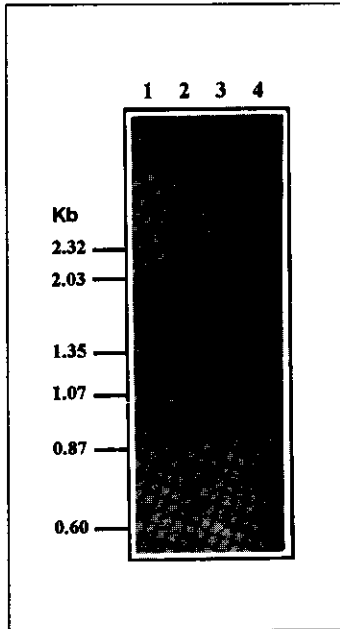
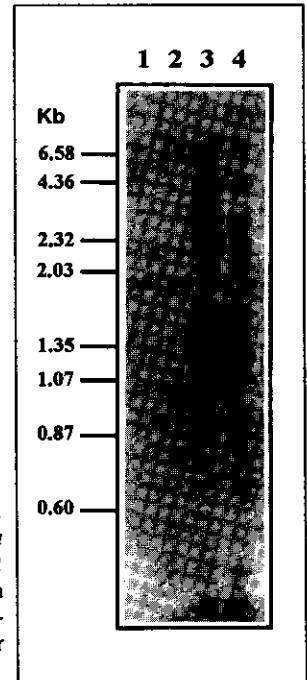


Fig. 4. Specificity of probe 1-me. *Sau3AI* partially digested *Agmasoma* DNA from *P. merguensis* (lane 1), *Agmasoma* DNA from *P. monodon* (lane 2), *P. merguensis* DNA (lane 3) and *P. monodon* DNA (lane 4) were blotted onto the filter and probed with dATP³²P labelled 1-me. The probe hybridized with equal visual intensity to *Agmasoma* DNA from either shrimp source but not with host shrimp DNA.

Fig. 5. Specificity of probe 1-mo. *Sau3AI* partially digested *P. merguensis* DNA (lane 1), *P. monodon* DNA (lane 2), *Agmasoma* DNA from *P. merguensis* (lane 3) and *Agmasoma* DNA from *P. monodon* (lane 4), were blotted onto the filter and probed with dATP³²P labelled 1-mo. The probe hybridized with equal visual intensity and with the same pattern to *Agmasoma* DNA from either shrimp source but not with host shrimp DNA.



Discussion

The method described for purification of *Agmasoma* spores from infected natural tissue effectively removed contaminating host cells and possible bacterial contaminants. The percentage of recovery was not high, but this did not pose a problem because of the high quantity of spores present in infected animals. The technique was convenient, did not require complicated instruments and could be completed in 2 hours. The method was less expensive than those previously reported for isolating other kinds of microsporidia using ludox or percoll solutions (Undeen and Avery 1983) and did not result in germination of the spores. The isolation of the *Agmasoma* spores was probably facilitated by the fact that cells of the marine shrimp quickly lysed in the hypotonic distilled water used for spore preparation.

Although complete removal of host material could not be absolutely proven, subsequent results from *in situ* colony hybridization indicated that the technique resulted in good removal of potentially contaminating host DNA. From 200 transformed colonies randomly selected for construction of an *Agmasoma* DNA library, no clone expressed a strong autoradiographic signal to the dATP³²P labelled host shrimp DNA.

The *Agmasoma* DNA from each shrimp host was isolated and cloned at different times. Randomly selected candidate probes derived from each were shown by slot blot hybridization to hybridize with DNA of the other. In addition, identical patterns were obtained from autoradiographs of Sau3AI partially digested *Agmasoma* DNA from either shrimp species when they were probed with dATP³²P labelled DNA fragments derived from either shrimp species. That is, probe 1-me from *P. merguensis* gave a pattern consisting of one strong band that was identical with *Agmasoma* DNA digests from both species of shrimp, and 1-mo from *P. monodon* gave a different pattern of many strong bands that was also identical with *Agmasoma* DNA digests from both species of shrimp. This indicated a close relationship between the DNA from the two sources and supports the contention that a single parasite infects both species of shrimp (Flegel et al. 1992). The many autoradiographic signals found for probe 1-mo could be interpreted as evidence that the cloned DNA fragment was derived from a multicopy sequence. In addition, it was shown from dot blot hybridization tests using diluted *Agmasoma* DNA hybridized with DIG labelled 1-mo and DIG labelled amplified small subunit rRNA (SSU rRNA) probe (Pasharawipas et al. 1994) that the 1-mo was more sensitive than the rRNA probe. Since the SSU rRNA gene is a multicopy gene, this data also supported the contention that probe 1-mo was derived from a multicopy sequence.

Since it was inconvenient to sequence the whole recombinant plasmid, it was subcloned to 736 base pairs from the 5' end and the subcloned probe, 1.1-mo, was found to have essentially the same specificity and sensitivity as the original 1-mo probe. Sequencing showed that the probe was composed mainly of AT bases (66% in 736 base pairs). Using couple primers designed from the 1.1-mo nucleotide sequence, samples from both host shrimp, from *E. coli* and *V. parahaemolyticus* and from some fish species were subjected to PCR amplification (Innis and Gelfand 1990). Agarose gel electrophoresis showed that only DNA from *Agmasoma* could be amplified (data not shown).

The probes 1-mo and 1.1-mo were selected for further tests aimed at discovering the intermediate host of *Agmasoma* and tracing steps in the life cycle of the parasite even in the absence of recognizable morphological forms. In preliminary tests, 22 organisms were collected from a shrimp-farm water supply canal where *P. merguensis* infected with *Agmasoma* was present. Using slot blot hybridization with crude DNA extracts, DNA from two fish species only showed a positive reaction with probe 1-mo. These fish were *Priacanthus tayenus* and *Scatophagus argus* (Matchacheep 1992). Later, a DNA extract of both fish also gave an amplified product under PCR using 1.1-mo couple primers, and this product hybridized strongly with 1-mo in Southern blot hybridizations. Bioassays are now underway with the suspect fish to determine whether they can act directly as intermediate hosts for this shrimp parasite. It is also hoped that the probe will be useful for the early detection of epidemic infections in shrimp.

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