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Lisa M. Ragone Calvo
Virginia Institute of Marine Science

Nancy A. Stokes
Virginia Institute of Marine Science

Eugene Burreson
Virginia Institute of Marine Science

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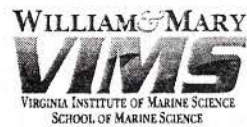
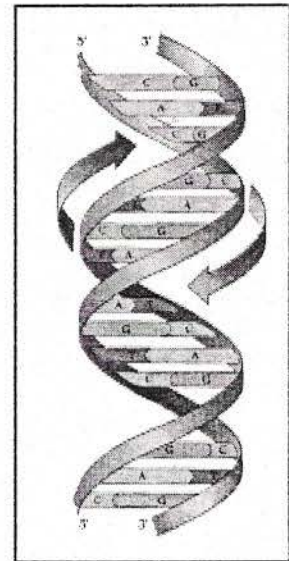
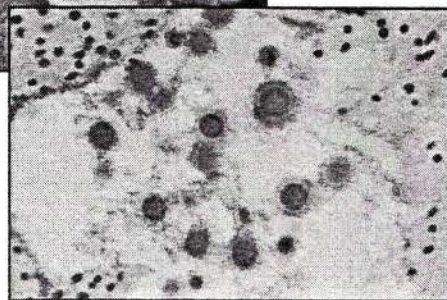
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School of Marine Science
Virginia Institute of Marine Science
College of William and Mary
Gloucester Point, Virginia 23062



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Introduction

Quahog parasite unknown, QPX, is an emerging disease of hard clams, *Mercenaria mercenaria*, that has been documented to be present in Massachusetts, New Jersey, and Virginia, USA and Prince Edward Island, Nova Scotia, and New Brunswick, Canada. The protozoan parasite is primarily found in clams older than about 1.5 years and has caused severe clam mortalities (>80%) in some areas. The swiftness by which epizootics can emerge and their potentially devastating impact necessitate the development of effective disease management strategies for both traditional fisheries and aquaculture. Rapid, sensitive, and accurate diagnosis of disease agents is a critical component of any disease management strategy. At the present time, diagnosis of QPX has been achieved using standard histological techniques. Here we present alternative, molecular-based diagnostic methodologies for the detection of QPX.

The first technique employs polymerase chain reactions (PCR) using QPX specific primers and the second method, in situ hybridization, utilizes QPX specific DNA probes that are applied to paraffin-embedded tissue sections. Validation studies of PCR-based diagnosis of QPX with standard histological methods indicate equivalent sensitivity when infections are multifocal and light to moderate in intensity. In cases of focal and rare to light intensity infections, standard histological examination was more sensitive; however, the sensitivity of PCR can be increased by replicate sampling of tissues. In situ hybridization, while not practical for disease monitoring purposes, offers an excellent means for verifying the identity of QPX in histological tissue sections.

1.0 PCR-based detection of QPX

Introduction

The polymerase chain reaction provides a rapid method for amplifying relatively small regions (generally <2000 base pairs) of DNA. Two short oligonucleotide primers (20-30 bp), one corresponding to each strand of the DNA double helix, are designed to flank a specific target or template region of DNA. The primers must be specific to the particular sequence and not anneal to any other non-target DNA sequence. Using PCR the region between the two primers can be repeatedly copied, with each new copy serving as an additional template. By this method the number of target sequences doubles during each round of replication and the nucleotide sequence can be amplified a billionfold. This amplification enhances the ability to detect small amounts of DNA. In a typical round, or cycle, the reaction mixture, which contains the genomic DNA, the primers, DNA polymerase, and four deoxyribonucleoside triphosphates, is first heated to denature the double stranded genomic DNA. The reaction mixture is then cooled allowing the primers to anneal to complementary sequences in the genomic DNA. The mixture is subsequently heated and DNA polymerase catalyzes the synthesis of DNA in regions downstream from each of the two primers. This cycle is generally repeated around 30 times and the amplification product is then detected by electrophoresis. The QPX primers utilized here, QPX-F and QPX-R2, specifically amplify a 665 bp region of the QPX small subunit ribosomal DNA (SSU rDNA).

1.1 Isolation of DNA from clam tissues

Methods for the extraction of DNA from clam tissues were developed using an adaptation of the guanidine thiocyanate purification method of Hill et al., 1991.

Reagents

5.9 M Guanidine Thiocyanate (GTC)

6.9714 g GTC in dH₂O to total volume of 10 ml

Warm tube to get GTC into solution. For storage, wrap in aluminum foil; discard solution if it turns yellow.

TE Buffer

5 ml 1M Tris-HCl buffer, pH 8.0

1 ml 0.5 M EDTA

494 ml distilled water

1 M Tris-HCl Buffer (tris(hydroxymethyl)aminoethane)

121 g Tris base

800 ml dH₂O

Adjust pH to 8.0 with concentrated HCl

Add dH₂O to 1 liter

Filter to remove particulates and autoclave

0.5 M EDTA (ethylenediamine tetraacetic acid)
18.61 g $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$
700 ml dH_2O
Adjust pH to 8.0 with 10 M NaOH
(EDTA will not go into solution until pH is close to 8.0)
Add dH_2O to 1 liter
Filter to remove particulates and autoclave
Store at 4°C

3 M sodium acetate (NaOAc)
408 g sodium acetate $\cdot 3\text{H}_2\text{O}$
400 ml dH_2O
Adjust pH to 5.2 with acetic acid
Add dH_2O to 1 liter
Filter to remove particulates and autoclave

Chloroform

Isopropanol

Procedure

1. Carefully shuck clams. With clean scalpel dissect 0.125 g mantle tissue and place in 1.5 ml capacity microcentrifuge tube. Clean scalpel with 95% ethanol and flame between each sample to avoid carry-over contamination.
2. Add 0.25 ml TE buffer and carefully homogenize tissue with sterile grinder.
3. Spin in microcentrifuge at 14,000 x g for 4 min.
4. Decant supernatant and add 25 μl 5.9 M GTC to pellet. Stir with pipet tip to mix.
5. Incubate at 60°C 90 min.
6. Add 475 μl sterile dH_2O and invert to mix.
7. Extract lysate by adding an equal volume of chloroform. Mix well by inverting tube.
8. Spin in microcentrifuge at 14,000 x g for 5 min.
9. Take top layer (transfer to new microcentrifuge tube) and extract by adding an equal volume of chloroform. (Bottom layer will be chloroform and tissue debris will be at interface.)
10. Spin in microcentrifuge at 14,000 x g 5 min.
11. Take top layer and precipitate with 0.1 volume 3 M NaOAc and 0.6 volume isopropanol (i.e. for 550 μl lysate volume add 55 μl and 330 μl , respectively).
12. Gently invert tube a few times. Store at -20°C at least 2 hours.
13. Spin in microcentrifuge at 4°C, at 14,000 x g 15-20 min.
14. Remove supernatant, add 0.5 ml 70% ethanol, and gently invert tube a few times.
15. Spin in microcentrifuge at 14,000 x g 2 min.
16. Remove supernatant and air dry pellet at room temperature 1-2 hr.
17. Add 100 μl TE buffer. Do not mix, allow to resuspend overnight at 4°C.
18. Isolated DNA can be stored for up to 1 month at 4°C; for longer term storage, hold at -20°C.

Note: Wear gloves and work in fume hood when working with chloroform.

Comments

1. In recent trials QIAamp® DNA Mini Kit (Qiagen Inc., Valencia CA) has also successfully been utilized for isolation of DNA from clam tissues. Dissect 50 mg clam mantle tissue and place in 1.5 ml microcentrifuge tube. Directly follow kit instructions, using modifications for 50 mg starting material. Allow the 56°C incubation to proceed until tissue is completely lysed (about 18 hours).

1.2 DNA Amplification

Reagents

sterile dH₂O (stock tube used only for PCR)

Bovine Serum Albumin (BSA), 1 mg/ml solution in dH₂O

dNTP solution

20 µl 10 mM dATP

20 µl 10 mM dCTP

20 µl 10 mM dGTP

20 µl 10 mM dTTP

5X buffer D (see comment 1)

TE Buffer (see Reagents Section 1.1)

Stock tube used only for PCR

Primer QPX-F

1 pmole/µl solution in TE Buffer (see comment 2)

Primer QPX-R2

1 pmole/µl solution in TE Buffer (see comment 2)

Taq DNA polymerase (5 units/µl)

Procedure

1. Prepare master mix. Based on proportions listed below calculate the total volume of each reagent needed for the total number of reactions that will be run (see comment 3). Combine reagents in the order listed, maintain reaction components on ice and prepare immediately before use.

Amount per reaction

- 6.63 μ l sterile dH₂O
- 10 μ l BSA
- 5 μ l 5X buffer D
- 2 μ l dNTP
- 0.125 μ l primer QPX-F
- 0.125 μ l primer QPX-R2
- 0.12 μ l Taq DNA polymerase

After preparing master mix, stir or pipet up and down to mix components.

2. Aliquot 24 μ l of the master mix to each sample tube.
3. Add 1 μ l template DNA (isolated as described above), mix by stirring or pipetting, and then place tube on ice until all samples are ready to load into the thermal cycler. Mix DNA by tapping tube with finger before pipetting into PCR reaction.
4. Place in thermocycler using the following schedule:

<u>Step</u>	<u>Temperature</u>	<u>Time</u>
1. Initial melt	94°C	4 min
2. Denature	94°C	30 sec
3. Anneal	56°C	30 sec
4. Extend	72°C	1.5 min
5. Cycle steps 2-4 35 times		
6. Final Extension	72°C	5 min
7. Hold	4°C	forever

5. Estimate yield by running a 2.5 μ l aliquot of the PCR reaction on an agarose gel (1.5%) following standard electrophoresis procedures. A 100 base pair ladder, or other suitable DNA marker, should be used as a reference.
6. Stain the gel by soaking in 1 μ g/ml ethidium bromide solution for 30 min and photograph in long wave (301 nm) UV.

Ethidium bromide is carcinogenic; wear nitrile gloves when in contact with the solution or stained gels.

Wear safety glasses when UV light is on.

7. Positive QPX samples will yield a 665 bp product (Figure 1).
8. Reamplify for detection of light and rare infections (see comment 5).

Comments

1. 5X buffer D is available from Invitrogen, Carlsbad, CA 92008.
2. Sequences of the QPX-F and QPX-R2 primers are as follows:
QPX-F (5'-ATCCTCGGCCTGCTTTTAGTAG-3')
QPX-R2 (5'-GAAGTCTCTACCTTTCTTGCGA-3')
Primers can be purchased from a number of biotech companies specializing in primer and probe synthesis. The commercially synthesized primers will be sent lyophilized. Resuspend primers to 100 pmol/ μ l in TE (maintain separate stock of this buffer for PCR), aliquot, and store at -20°C .
3. Prepare enough master mix for all unknown sample reactions plus one negative control (add dH_2O instead of DNA to reaction), one positive control (QPX SSU rDNA plasmid, 1 pg/ μ l) and one extra per ten reactions to allow for pipetting error. Use aerosol resistant ("filter") pipet tips to prevent contamination between reactions.
4. Store all PCR reagents, except dH_2O , at -20°C . Aliquot all reagents to reduce repetitive freeze/thaws and prevent contamination.
5. To reamplify, follow above PCR amplification procedure using 1 μ l of the previously amplified reaction mix as template DNA in step 3.

Literature Cited

Hill, W. E., S. P. Keasler, M. W. Trucksess, P. Feng, C. A. Kaysner, K. A. Lampel. 1991. Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Applied and Environmental Microbiology* 57:707-711.

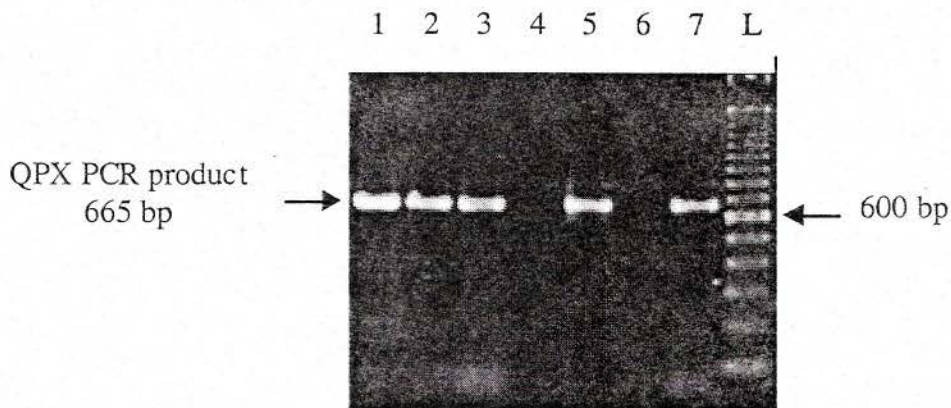


Figure 1. QPX PCR amplification products on 1.5% agarose gel. Lanes 1-3, 4, and 5 are QPX PCR reactions of DNA from respectively, cultured QPX cells, an uninfected clam, and an infected clam. Samples positive for QPX show a 665 bp product. Lane 6 is negative control (no DNA), lane 7 is positive control, and lane L is 100 bp DNA ladder.

2.0 In Situ Hybridization of DNA probes for the detection of QPX in paraffin-embedded tissue sections

Introduction

The application of DNA probes with in situ hybridization (ISH) can be used to detect target DNA or RNA in a particular sample. DNA probes are designed to contain a sequence that is specific to a particular target organism or gene. When the probe is applied to the sample it hybridizes to its complementary sequence, which is found only in the cells of interest. A colorimetric or fluorescent label is attached to the probe rendering the hybridized DNA readily detectable. For disease diagnosis, in situ hybridizations are generally performed on paraffin-embedded tissue sections. The specific labeling of the target DNA with the probe facilitates detection and localization of the disease agent.

The following protocol employs two oligonucleotide probes that have sequences unique to QPX. The commercially synthesized probes are labeled with digoxigenin and applied to paraffin-embedded tissue sections. Its application is most appropriate for processing small numbers of samples for verification of putative QPX or for the detection and localization of the parasite when infection intensities are very low. The QPX probes target the small subunit ribosomal RNA gene. When applied to a tissue section containing QPX, the probes hybridize to their complementary sequences, which are in the DNA encoding the gene and the ribosomal RNA. These complementary sequences are present in both the nucleus and cytoplasm of the QPX cells. Visualization of the hybridized probes is accomplished through the application of an anti-digoxigenin antibody, which is coupled to an enzyme, alkaline phosphatase, that yields a purplish-black color upon treatment with a solution of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP). The labeled cells are easily detected using light microscopy.

2.1 Preparation of histological sections

The in situ hybridization method presented here was developed using clam tissues that were preserved in Davidson's AFA or neutral buffered formalin. For histological processing follow standard techniques. Sections of paraffin-embedded tissues should be 5-6 μm thick and should be mounted on positively-charged microscope slides. To avoid carry-over contamination, microtome blades and forceps should be cleaned with xylene between each sample.

2.2 In situ hybridization

Reagents

Proteinase K stock solution (10 mg/ml)

10 mg Proteinase K

10 ml dH₂O

Aliquot and store at -20°C

Proteinase K working solution (50 µg/ml)
90 µl of 10 mg/ml proteinase K stock
17.91 ml PBS (warm to 37°C before combining)

Phosphate buffered saline (PBS)

8.0 g NaCl
0.2 g KCl
1.44 g Na₂HPO₄
0.24 g KH₂PO₄
800 ml dH₂O
pH to 7.4 with HCl
Adjust to 1 liter with dH₂O, filter to remove particulates

0.2% Glycine

2.0 g glycine
1 liter PBS
Autoclave

20X SSC

175 g NaCl
88 g Na₃citrate•2H₂O
700 ml dH₂O
pH to 7.0 with 1 M HCl
Adjust to 1 liter with dH₂O, filter to remove particulates

50X Denhart's (Purchased as pre-made solution)

Prehybridization solution, 50 ml

5.0 ml distilled water
10.0 ml 20X SSC
25.0 ml formamide
5.0 ml 50X Denhardt's
2.5 ml 10 mg/ml yeast tRNA
2.5 ml 10 mg/ml salmon sperm DNA
Immediately before use, heat prehybridization solution in boiling water bath for 10 min, quickly cool by placing on ice for several minutes (see comment 1).

Hybridization solution

Follow hybridization solution recipe with the following alterations: 1) add 1.0 ml dH₂O instead of 5.0 ml, and 2) it is not necessary to heat before use. Store at -20°C.

QPX probe hybridization solution (4 ng/ μ l)

- 1 μ l digoxigenin-labeled oligonucleotide probe QPX641 (0.4 mg/ml stock)
- 1 μ l digoxigenin-labeled oligonucleotide probe QPX1318 (0.4 mg/ml stock)
- 98 μ l hybridization solution
- See comment 2 for more information on probes

4X SSC/50% formamide solution

- 20 ml 20X SSC
- 30 ml dH₂O
- 50 ml formamide

2X SSC

- 1:10 dilution of 20X SSC in dH₂O

1X SSC

- 1:20 dilution of 20X SSC in dH₂O

0.5X SSC

- 1:40 dilution of 20X SSC in dH₂O

Buffer 1 (100 mM Tris-HCl, 150 mM NaCl)

- 12.11 g Tris base
- 8.76 g NaCl
- 800 ml dH₂O
- pH to 7.5 with HCl
- Add dH₂O to 1 liter and filter

Blocking solution

- 15.20 ml Buffer 1
- 0.48 ml 10% Triton X-100
- 0.32 ml normal sheep serum

Antibody solution

- 960 μ l Buffer 1
- 30 μ l 10% Triton X-100
- 10 μ l normal sheep serum
- 2 μ l anti-digoxigenin alkaline phosphatase antibody conjugate

Buffer 2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂)

- 12.11 g Trisma base
- 5.844 g NaCl
- 10.164 g MgCl₂
- 800 ml dH₂O
- pH to 9.5 with HCl
- Add dH₂O to 1 liter and filter

Color Solution

15.71 ml Buffer 2
72 μ l 75 mg/ml nitroblue tetrazolium (NBT)
56 μ l 50 mg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) solution
160.0 μ l 24 mg/ml levamisole
Prepare immediately before use

TE Buffer (10 mM Tris-HCl, 1 mM EDTA)

10 ml 1M Tris-HCl buffer, pH 8.0
2 ml 0.5 M EDTA
988 ml distilled water

Tris-HCl Buffer (tris(hydroxymethyl)aminoethane)

121 g Tris base
800 ml dH₂O
Adjust pH to 8.0 with concentrated HCl
Add dH₂O to 1 liter
Filter to remove particulates and autoclave

0.5 M EDTA (ethylenediamine tetraacetic acid)

18.61 g Na₂EDTA.2H₂O in
700 ml dH₂O
Adjust pH to 8.0 with 10 M NaOH
Add dH₂O to 1 liter
Filter to remove particulates and autoclave
Store at 4°C

0.05% Bismarck Brown Y

0.5 g Bismarck Brown Y in 1 liter dH₂O
Filter stain through Whatman 4 filter paper before each use.
Stain can be used for about 1 year.

Procedure

2.2A Preparation of Tissue Sections

1. Dewax paraffin-embedded tissue sections in xylene for 10 min.
2. Rehydrate sections in:
 - 100% ethanol for 10 min, 2x
 - 95% ethanol for 1 min, 1x
 - 80% ethanol for 1 min, 1x
 - 70% ethanol for 1 min, 1x

3. Wash in PBS for 5 min, 2x (see comment 3)
4. Treat tissues with proteinase K to enable probe to penetrate the cells. Place slides in slide mailer box (see comment 4) containing 18 ml of 50 µg/ml proteinase K.
5. Incubate at 37°C for 15 min.
During proteinase K incubation, prepare prehybridization solution (ie. place in boiling water bath for 10 min, then cool on ice).
6. Wash slides with 0.2% glycine in PBS for 5 min at room temperature (RT) to stop the proteolysis.
7. Incubate slides in 2X SSC for 10 min at RT.

2.2B. Prehybridization

1. During 2X SSC incubation pipet 16 ml prehybridization solution into a slide mailer box and place in beaker inside 42°C incubator to prewarm the solution.
2. Place slides in slide mailer box and incubate for 1 hour, 42°C.

2.2C Hybridization

1. Prepare QPX probe hybridization solution.
2. Remove slides from incubator and carefully wipe off excess prehybridization solution. Be sure not to touch tissue.
3. Encircle tissue with PAP pen, apply 50 µl QPX probe hybridization solution within circle (don't touch tissue with pipet tip), and cover with plastic cover slip.
*If tissue section is quite large, apply 75 µl QPX probe hybridization solution.
4. Place slides on foil-covered heating block set at 86°-90°C for 12 min, then cool slides on ice for 1 min. If air bubbles develop over tissue section, carefully push them past PAP pen circle with fingernail or wooden applicator stick. Do not rub tissue.
5. Prepare humid chamber by placing a piece of absorbent paper on the bottom of a plastic, air-tight container. Add just enough 4X SSC/50% formamide solution to saturate the paper and place wooden applicator sticks on the wet paper.
6. Place slides on sticks in humid chamber, close lid, and incubate overnight at 42°C.
7. Remove plastic cover slips and wash the sections as follows:
2X SSC, 5 min, RT, 2x
1X SSC, 5 min, RT, 2x
0.5X SSC, 10 min, 42°C, 2x

2.2D Immunological Detection

1. Wash slides in Buffer 1 for 1 min.
2. Prepare 16 ml blocking solution in a slide mailer. Incubate slides in blocking solution for 30 min, RT.
3. Prepare 1 ml antibody solution.
4. Remove SSC/formamide-saturated paper and sticks from the humid chamber.
5. Place fresh piece of absorbent paper in humid chamber and saturate with water.
6. Reapply PAP pen circle around tissue, add 50 µl antibody solution to slide, cover with plastic cover-slip, and place slides in humid chamber. Close lid and incubate for 3 hours, RT.
7. Wash slides in Buffer 1 for 5 min, 2x.

8. Wash slides in Buffer 2 for 5 min, 2x.
9. Prepare 16 ml color solution in slide mailer box, add slides, and place slide mailer in beaker to keep it upright. Cover with foil and incubate for 2 hours.
Wear gloves when handling components of color solution.
10. Wash slides in TE buffer 5 min to stop color reaction.
11. Wash slides in dH₂O, 1 min.
12. Place slides in 0.05% Bismarck Brown Y and allow to stain for 3 min.
13. Rinse with 3 washes in dH₂O.
14. Coverslip using aqueous-based mounting medium.

Comments

1. Heating of prehybridization solution is required to denature salmon sperm DNA, which acts as a non-specific blocking agent. Prehybridization solution can be reused up to 5 times. If reusing, store at -20°C and heat and cool immediately before each use as described.
2. Sequences of the QPX641 and QPX1318 probes are as follows:
QPX641 (5'-GATGACACACAGCAAACATTGACAT-3')
QPX1318 (5'-GAAGTCTCTACCTTTCTTGCGA-3')
Probes can be purchased from a number of biotech companies specializing in primer and probe synthesis. When ordering probes request desalt and HPLC purification and a 5' digoxigenin modification. The commercially synthesized probes will be sent lyophilized. Resuspend probes to 0.4 mg/ml in TE, aliquot, and store at -20°C.
3. If processing a small number of slides (<6) washes may be carried out by placing slides on staining rack over sink and very gently flooding. For larger numbers, slides may be placed in a staining vessel filled with appropriate solution volume to cover slides.
4. Polypropylene microscope slide mailer boxes (such as Thomas Scientific, catalog no. 6707M27) work well for several steps of this protocol, namely protease treatment (2.2A4), prehybridization (2.2B), blocking (2.2D2), color detection (2.2D7). Each mailer holds 4 slides and 16-18 ml solution.
5. Hybridized cells appear purplish-black against golden brown stained tissue (Figure 2).

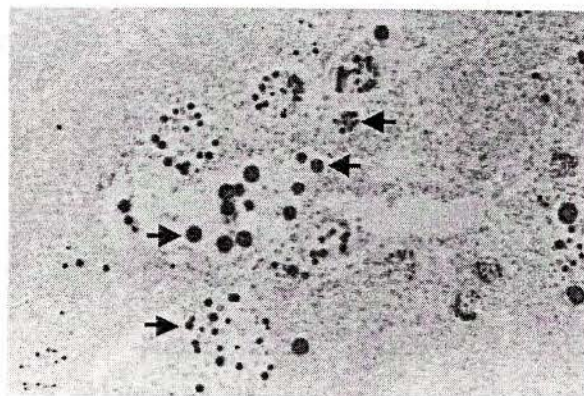


Figure 2. In situ hybridization of QPX cells in an infected clam. ISH of tissue section using 4 ng/μl each of DNA probes QPX641 and QPX1318. Arrows indicate some of the QPX cells, which are stained purplish-black.

