

5.2.1 *Perkinsus* spp. Infections of Marine Molluscs (2020)

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A. Name of Disease and Etiological Agents

Following the earliest reports of infections by the previously unknown pathogen *Perkinsus marinus* in the eastern oyster *Crassostrea virginica* of the Gulf of Mexico (Mackin et al. 1950), the name of the disease was colloquially abbreviated as Dermo in reference to the obsolete initial name of that pathogen, *Dermocystidium marinum* (= *Labyrinthomyxa marina*). Both the geographic and host ranges of *P. marinus* have expanded since its description as the first member of a genus that has grown substantially. Since 1981, additional *Perkinsus* spp. parasites and diseases have been described and reported in a variety of bivalve and gastropod marine molluscs. Other current *Perkinsus* species include *P. olseni* (= *P. atlanticus*) from Australian abalone *Haliotis* spp. and a wide variety of bivalves worldwide; *P. chesapeaki* (= *P. andrewsi*) from clams, cockles, and oysters of mid-Atlantic USA, Europe, Australia, and Brazil; *P. mediterraneus* from *Ostrea edulis* oysters and other bivalves of the Mediterranean Sea; *P. honshuensis* from clams of Japan and Korea; *P. beihaiensis* from oysters and clams of China, India, and Brazil, and *P. qugwadi* (*incertae sedis*) from scallops of Pacific Canada. With seven *Perkinsus* species described now in 2020, the generic disease name perkinsosis is most appropriate for all.

Current phylogenetic analyses consistently place the genus *Perkinsus* among the alveolate protists, basal to the dinoflagellates (Siddall et al. 1997, Saldarriaga et al. 2003, Bachvaroff et al. 2011); with

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general support for a proposed phylum Perkinsozoa. Phylogenetically, *P. qugwadi* is basal to the other species of the genus *Perkinsus*. Based on current genetic data (i.e. SSU rRNA gene and ITS region sequences), it is 4 times to >10 ten times more distant from the other *Perkinsus* species than they are from each other, making its placement in the genus tenuous (Blackbourn et al. 1998, Itoh et al. 2013).

B. Known Geographic and Host Ranges of the Diseases

Perkinsus marinus (Mackin et al. 1950) infections (dermo disease) occur widely in the oyster *C. virginica* of the Gulf of Mexico and Atlantic coasts of North America, from Campeche, Mexico to Maine, USA. The pathogen also infects *C. virginica* of Hawaii (Kern et al. 1973), *Crassostrea* and *Saccostrea* spp. of the Gulf of California (Càceres-Martinez et al. 2012), and *Crassostrea* spp. of Brazil (da Silva et al. 2013). It also occasionally infects clams of USA Atlantic coasts, including *Mercenaria mercenaria* (Ray 1954a), *Mya arenaria* (Reece et al. 2008), and *Macoma balthica* (Coss et al. 2001). *Perkinsus marinus* is a reportable pathogen in jurisdictions regulated by the World Organisation for Animal Health (OIE 2019a) or by the U.S. Department of Agriculture (USDA-APHIS 2020).

Perkinsus olseni (Lester and Davis 1981) (= *P. atlanticus*, Azevedo 1989) infections are reported in Australian abalones *Haliotis rubra*, *H. laevigata*, *H. cyclobates*, and *H. scalaris*. Parasites of the same species infect diverse bivalve molluscs of a broad geographic range, including clams, cockles, pearl oysters, and oysters of Australia, New Zealand, Thailand, China, Japan, Korea, India, Italy, Spain, Portugal, France, Uruguay, and Brazil (Murrell et al. 2002, Villalba et al. 2004, Arzul et al. 2012, da Silva et al. 2014). *Perkinsus olseni* is a reportable pathogen in jurisdictions regulated by the World Organisation for Animal Health (OIE 2019b) or by the U.S. Department of Agriculture (USDA-APHIS 2020).

Perkinsus chesapeaki (McLaughlin et al. 2000) (= *P. andrewsi*, Coss et al. 2001) infections are reported at high prevalences in the clams *Mya arenaria*, *Tagelus plebeius*, *Macoma balthica*, and others of Chesapeake and Delaware bays (Burreson et al. 2005, Reece et al. 2008). It also infects clams of *Ruditapes* (= *Venerupis*) species of both Atlantic and Mediterranean waters of Europe (Arzul et al. 2012, Ramilo et al. 2016), oysters *Crassostrea rhizophorae* of northeast Brazil (Neto et al. 2016), and cockles *Anadara trapezia* of eastern Australia (Reece et al. 2017).

Perkinsus mediterraneus (Casas et al. 2008) infects wild and cultured flat oysters *Ostrea edulis*, as well as clams and scallops, from the western Mediterranean Balearic Islands (Valencia et al. 2014).

Perkinsus honshuensis (Dungan and Reece 2006) infects clams of the *Ruditapes* species in Japan (Umeda and Yoshinaga 2012) and Korea (Kang et al. 2016).

Perkinsus beihaiensis (Moss et al. 2008) infects oysters and clams of China and Brazil (Sabry et al. 2013, Ferreira et al. 2015, Cui et al. 2018) and oysters of India (Sanil et al. 2012).

Perkinsus qugwadi (*incertae sedis*) (Blackbourn et al. 1998) infects cultured Japanese scallops, *Mizuhopecten* (= *Patinopecten*) *yessoensis*, of Canadian Pacific waters, where infections were intermittently detected during 1988-1997, and again in 2011 (Itoh et al. 2013).

Sequences of PCR amplicons from DNAs extracted from tissue biopsies of diverse molluscs from both the Caribbean and Pacific coasts of Panama identified *P. beihaiensis*, *P. marinus*, *P. olseni*, and

P. chesapeaki, but histological analyses were not performed to confirm infections or lesions among the surveyed Panamanian molluscs (Pagenkopp Lohan et al. 2016, Pagenkopp Lohan et al. 2018).

C. Epizootiology

Although *P. marinus* infections occur and persist among *C. virginica* from waters with temperatures as low as 2 °C and salinities as low as 3 ‰, parasite proliferation and disease impacts are greatest among oysters from waters in the broad upper ranges of temperature (20-30 °C) and salinity (10-25 ‰) that often occur together in Chesapeake Bay during late summer and autumn. Those are annual seasons when oyster mortalities from dermo disease peak, especially during drought years with elevated water salinities (Burrenson and Ragone Calvo 1996). *Perkinsus marinus* is transmitted directly between oysters through shared waters (Ray 1954a), and over distances ≥ 5 km (McCollough et al. 2007). Young juveniles and adult oysters may respectively acquire infections during 10-15 days of natural exposure (Ragone Calvo et al. 2003), and adult oysters may die from overwhelming acquired infections in as few as six weeks (Ray 1954b). In Gulf of Mexico waters, the ectoparasitic gastropod *Boonea impressa* serves as an active vector by transmitting parasite cells between oysters on which it feeds hypodermically (White et al. 1987).

Seasonal fluctuations in environmental temperatures and salinities may allow infected oysters to eliminate *P. marinus* infections, or to substantially reduce their intensities during seasons with physical conditions that limit parasite proliferation. Similar seasonal cycles are known with less detail for *P. chesapeaki* infections of Chesapeake Bay clams, while elevated prevalences and intensities for *Perkinsus* sp. infections are broadly documented during seasons of elevated water temperatures or salinities, among other molluscs of temperate or subtropical waters (Villalba et al. 2004, da Silva et al. 2014, Waki et al. 2018).

D. Disease signs

Behavioral changes, external gross signs, internal gross signs, and histopathological changes vary among different *Perkinsus* species and their diverse mollusc hosts. Consistent pathognomonic histological features of *Perkinsus* spp. within tissues of infected hosts include vacuolated, spherical parasite trophozoites that may show eccentric nuclei with prominent nucleoli (signet ring cells), as well as subdividing schizont cells containing multiple developing progeny trophozoites. Only *P. qugwadi* (*incertae sedis*) is described to produce and disseminate flagellated zoospores within tissues of its only known host, the Japanese scallop *Mizuhopecten* (= *Patinopecten*) *yessoensis* (Blackbourn et al. 1998, Itoh et al. 2013).

In *C. virginica*, reduced growth may precede mortality of *P. marinus*-infected oysters, and non-specific behavioral and gross signs include valve gaping, emaciation, and pale digestive glands (Ray and Chandler 1955, Paynter and Burrenson 1991).

Histological presentations of *P. marinus* in oysters include vacuolated, spherical parasite trophozoites with diameters of 2-20 μm that may show eccentric nuclei with prominent nucleoli (signet ring cells) (Figure 3), as well as subdividing, schizont cells with diameters of 5-20 μm that may show multiple nuclei of developing progeny. Digestive system epithelia of the intestine, digestive gland distributing ducts, and stomach are often heavily colonized and damaged. Oyster defensive responses include pronounced hemocyte infiltration of infected tissues (Figure 1), with frequent phagocytosis of parasite

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cells. Phagocytized parasite cells may be distributed systemically within circulating hemocytes, where they may also proliferate (Mackin 1951).

Perkinsus olsenii-infected Australian *Haliotis* spp. abalones, and venerid clams of Japan, Korea, and Spain, may show soft, white abscess nodules on surfaces of the foot, mantle, or gills (Goggin and Lester 1995, Camino Ordás et al. 2001, Choi and Park 2010). Infected Manila clams *Ruditapes philippinarum* may also show compromised burrowing abilities (Waki and Yoshinaga 2018).

Perkinsus spp. infecting clams of Chesapeake Bay and elsewhere show cytological features like those of *P. marinus*. However, they predominantly occur among connective tissues, especially of the gills, rather than digestive or other epithelia. Host responses in clams include hemocyte infiltration of infected tissues, phagocytosis of parasite cells, and hemocyte encapsulation of proliferating parasites (McLaughlin and Faisal 1998, Camino Ordás et al. 2001).

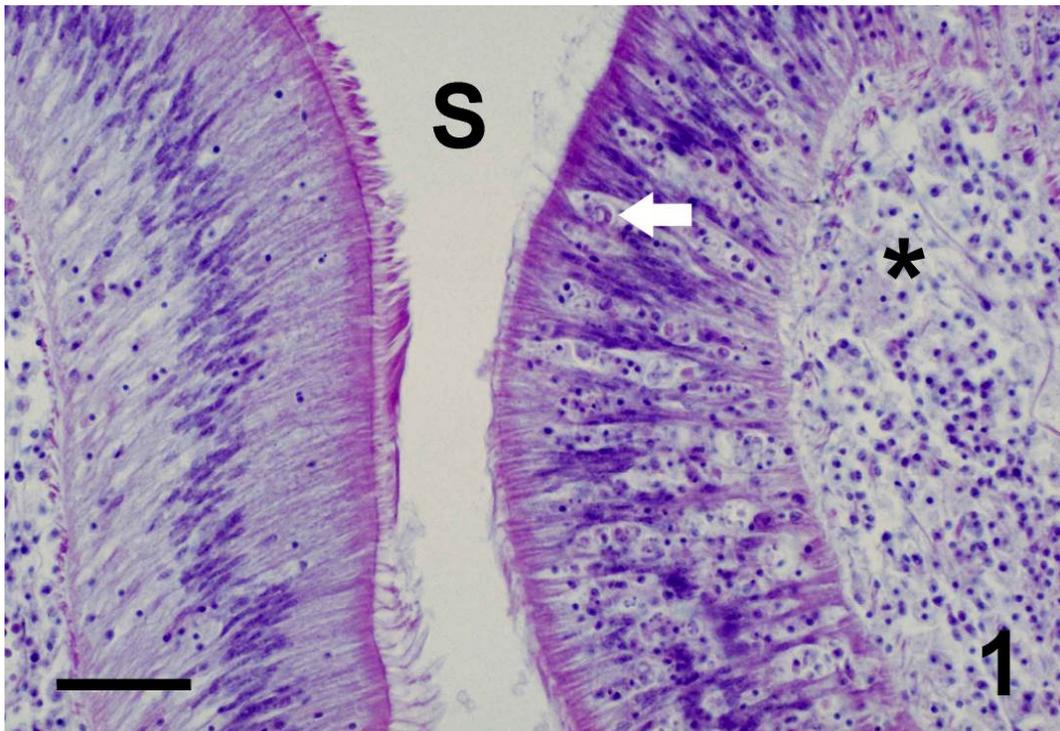


Figure 1. Ciliated epithelia lining opposite sides of the central lumen of an oyster stomach (S) are heavily infected by *P. marinus* (right) or nearly uninfected (left). Hematoxylin and eosin staining (H&E) shows numerous parasite trophozoites (arrow) colonizing the infected epithelium, which do not contrast strongly with surrounding host tissues. The uninfected epithelium (left) shows normal columnar architecture, with a mid-height band of nuclei and dense apical cilia. The infected epithelium (right) shows disrupted epithelial architecture, with necrotic abscesses where parasites proliferate among epithelial cells. Hemocytes prominently infiltrate the infected epithelium and its adjacent connective tissues (*). Bar = 50 μ m. From Reece and Dungan (2006) with permission.

E. Disease Diagnostic Procedures

Diagnostic procedures for detection and identification of *Perkinsus* spp. pathogens in tissues of marine and estuarine molluscs are listed for comparison in Table 1, with the qualifications that follow:

- Relative assay sensitivities are ranked numerically (0-4), based on published reports through 2019.
- Assays listed as genus-specific offer confirmatory diagnoses only to the genus level.
- Specificities of serological, PCR, and ISH assays depend on the documented specificities of their respective antibodies, primers, or probes.
- PCR assays detect targeted pathogen genomic DNAs extracted from host tissues, but they do not confirm the presence of live pathogen cells, active lesions, or infections.
- Due to the small tissue subsample volumes inherent to histological sections, histopathological methods were excluded from the highest sensitivity category.

Table 1. General attributes of diagnostic methods for *Perkinsus* spp. infections.

Method	Relative sensitivity ¹	Pathogen specificity	Presumptive diagnosis ²	Confirmatory diagnosis ²
Gross signs	0		-	-
Histopathology	1-3	genus	++	++
Immunohistochemistry	3	genus	++	++
ELISA assays	3-4	genus	++	+
RFTM assays				
1. hemolymph	1-3	genus*	++	+
2. solid tissue subsample	1-3		++	+
3. whole body burden	4		+++	+
PCR assays	3-4	genus* or species	+++ +++	+++ +++
ISH assays	3-4	genus* or species	+++ +++	+++ +++

¹ 0-4 increasing sensitivity

² applicability: - unsuitable, + limited utility, ++ standard method, +++ recommended method

* except *Perkinsus qugwadi*

1. Ray's high-salt fluid thioglycollate medium (RFTM) assay

Based on extensive testing to date, RFTM assays are considered to be specific for all members of the genus *Perkinsus* except *P. qugwadi* (*incertae sedis*) (Dungan and Bushek 2015). However, the apparent exclusivity of RFTM assays for detection of only *Perkinsus* species lacks exhaustive confirmation through testing of all other protists.

During incubation in RFTM for days, cells of *Perkinsus* species infecting host tissue samples enlarge dramatically (500 - 60,000%) without proliferation. Resulting enlarged, spherical parasite hypnospores or pre-zoosporangia measure 10-250 μm , with optically refractile walls that also stain blue-black in 20-30% (v/v) Lugol's iodine by a reaction that does not involve starch (Figure 2). Alternative Ray's fluid thioglycollate media (ARFTM) that lack agar and includes additional metabolites are superior for some applications (La Peyre et al. 2003), though ARFTM and RFTM media are treated as equivalent and synonymous in the protocols that follow.

Possible host tissue inocula and analytes include solid tissue sub-samples, hemolymph, and entire host bodies. Choices depend on the desired level of sensitivity, the required quality of quantitative results, and the tolerance for lethal sampling. Based on their relatively low costs, broad specificity, and substantial sensitivities, RFTM assays in several formats have been effectively used worldwide as both screening and diagnostic assays for many decades. Their genus specificity is adequate in circumstances where multiple *Perkinsus* species do not occur, or where diagnostic species identifications are not required. Strong correlations are reported between sample prevalences and infection intensities of individual oysters that were analyzed in parallel by RFTM assays and several qPCR assays (Yarnall et al. 2000, Gauthier et al. 2006). For RFTM methods and reviews, see Ray (1966), Bushek et al. (1994), La Peyre et al. (2003), and Dungan and Bushek (2015).

For RFTM assays of mollusc tissue subsamples, optimum inoculum tissues are selected according to parasite tissue tropisms, where known. Rectum and/or mantle tissues are commonly used to test for *P. marinus* infections in oysters, and gill or labial palp tissues are commonly used to test for *Perkinsus* sp. infections in some clams.

The most sensitive RFTM assay, and the only fully quantitative format, is the whole body burden (WBB) assay (i. below). Weighed host tissue sub-samples can be evaluated similarly, with inherently reduced sensitivity. In all cases, wet weights of host tissues are recorded before their incubation at 20-27 °C in 30-50 volumes of RFTM for 4-7 d, with optional homogenization. Host tissues are then hydrolyzed in 2M NaOH at 60 °C, leaving killed, unlysed, enlarged parasite cells that are stained with 20-30% (v/v) Lugol's iodine (Figure 2a). Parasite cell counts are normalized to recorded host tissue wet weights, and sensitivities reach 1 parasite cell/host when host tissues are analyzed exhaustively (Bushek et al. 1994). The use of ARFTM facilitates differential centrifugation steps of these assays by eliminating agar, a medium component that does not dissolve during alkaline hydrolysis (La Peyre et al. 2003).

Since *Perkinsus* species are aerobes, anaerobic gradients in RFTM are not required.

RFTM media preparation

- a. Rehydrate fluid thioglycollate medium with dextrose (Difco 0256-several, BBL 01-140, Sigma A0465) in distilled water plus 20 g/L NaCl. Stir and heat to dissolve with minimal boiling. To avoid the agar component of the standard medium, prepare alternative fluid thioglycollate medium similarly, as ARFTM (Difco 225710, BBL 211651, others) (La Peyre et al. 2003, Dungan and Bushek 2015).

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- b. Aliquot RFTM to assay tubes or bulk containers. Autoclave to sterilize. If bulk RFTM medium is autoclaved, aliquot aseptically to sterile assay tubes or culture plate wells before use.
- c. Supplement autoclaved RFTM with 50 µg/ml chloramphenicol (mutagen), or to 100 U/ml penicillin and 100 µg/ml streptomycin.
- d. Before inoculation with tissue samples, supplement RFTM aliquots with 50-200 U/ml nystatin, aseptically applied as a surface overlay if practical.

RFTM assay inoculation and incubation

- e. Aseptically inoculate RFTM aliquots with solid tissue subsamples, hemolymph samples, or whole oyster tissues or homogenates, at ≤ 0.1 g tissue/3 ml RFTM.
- f. Cap or cover RFTM assay containers. Incubate at 20-28 °C for 7-5 days, respectively.

RFTM assay analyses

Examine preparations for smooth, spherical, enlarged hypnospore cells with diameters of 10-250 µm and refractile walls. Those enlarged cells stain blue-black with Lugol's iodine (Figure 2b). Staining may vary with iodine penetration, or through its chemical neutralization by the L-cystine and thioglycollate reducing agents of residual RFTM or ARFTM media. Distinguish possible pollen grains or iodine crystals by morphology.

- g. Tissue subsample assays. Transfer and macerate solid tissue sub-samples in pools of 20-30% Lugol's iodine on microscope slides, add additional drops of dilute Lugol's iodine to cover macerated tissue, and coverslip. Suspension samples (hemolymph) may be iodine-stained in microplate wells or on filter membranes. Microscopically count or rank abundances of blue-black *Perkinsus* sp. hypnospore cells (Dungan and Bushek 2015).

or

- h. Whole or partial body assays (WBB). Pellet suspended solids by centrifugation at 1,500 x g for 10 min. Conservatively aspirate and discard RFTM supernate. Hydrolyze oyster tissues or homogenates in 5-10 volumes of 2M NaOH at 60 °C for 1-6 h, vortexing every 30 min. until tissue lysis is complete.

Pellet parasite hypnospores by centrifugation at 1,500 x g for 10 min. Aspirate and discard NaOH supernate. Resuspend parasite cell pellet and wash 3x for 10 min. at 1,500 x g in 0.1M phosphate-buffered saline (PBS) + 0.5% (w/v) bovine serum albumin (BSA) (PBS/BSA). Resuspend the washed pellet in 1 ml of PBS/BSA.

Stain the entire cell suspension with 20% filtered Lugol's iodine for microscopic enumeration, or quantitatively dilute suspension subsamples in PBS/BSA to yield countable dilutions (100-400 cells) before staining and enumerating subsamples.

Microscopically count or estimate total parasite cells/oyster from enumerated sample dilution factors, and normalize total parasite cells/g. host tissue wet weight. Treat weighed tissue sub-samples similarly (Bushek et al. 1994, La Peyre et al. 2003, Dungan and Bushek 2015).

or

- i. Live cell isolation and isolate propagation. Using a microscope for contrasting live cells if available, examine unstained, RFTM-incubated tissues or homogenate suspensions for thick-walled, refractile, vacuolated, 10-250 µm spherical, enlarged parasite hypnospore

cells. Rank and select optimal live inocula for parasite in vitro propagation or other live cell applications (Dungan and Bushek 2015).

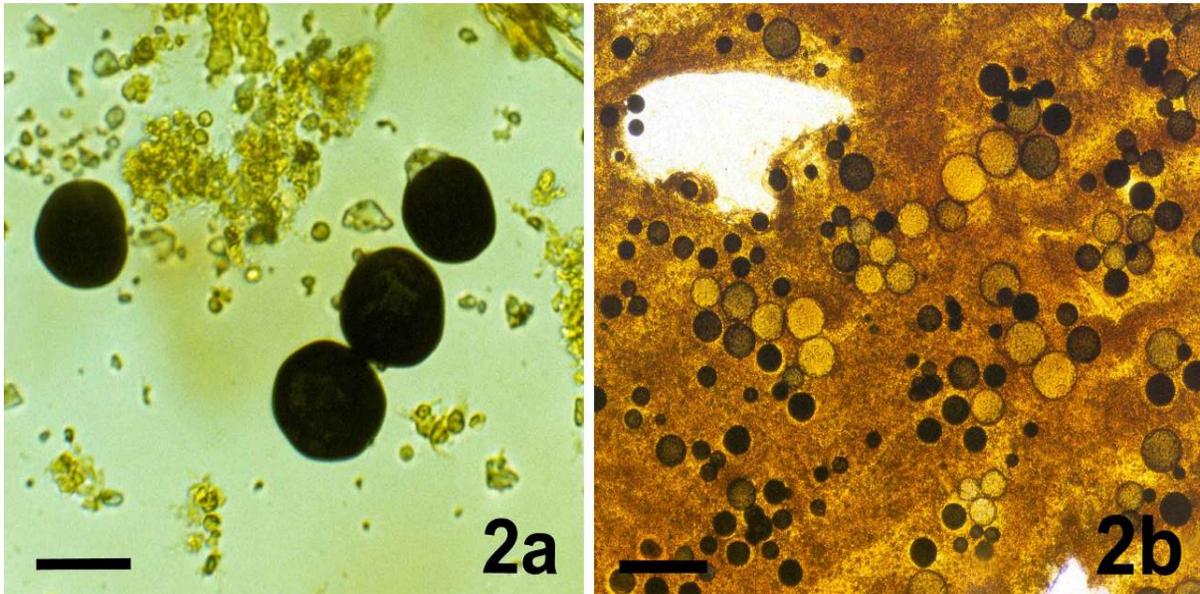


Figure 2. RFTM-enlarged *Perkinsus* sp. hypnospores stained with Lugol's iodine. **2a.** In whole body assay following NaOH hydrolysis of host oyster tissues. Bar = 50 μ m. **2b.** In macerated *Mya arenaria* labial palp tissues infected at moderate intensity. Incomplete iodine access, or neutralized activity, may leave some of the optically refractive parasite hypnospores lightly stained or unstained. Bar = 250 μ m. From Reece and Dungan (2006) with permission.

2. Histopathology

Their low chromatic contrast by H&E staining, along with their occasional small sizes, make detection of *Perkinsus* sp. cells in histological sections more challenging than in RFTM or other assays. Because individual histological sections include relatively small subsamples of analyzed host tissues, rare pathogen cells that occur in hosts bearing light infections or focal lesions may also be absent from analyzed sections. However, histological assays may match or surpass performances of other assays for some samples and circumstances. In tissues of most infected molluscs, the presence or activities of *Perkinsus* sp. infections often promote dramatic necrotic damage with evident inflammatory infiltration by host hemocytes in focal or systemic distributions (Figure 1). Especially among clams, proliferative foci of *Perkinsus* sp. cells may be prominently encapsulated by infiltrating hemocytes. Histological methods uniquely reveal parasite tissue tropisms and pathological effects, as well as host defensive responses. See **D. Disease Signs**.

3. Histological immunoassays

Fluorescent or colorimetric antibody labeling increases contrast of immunostained parasite cells and enhances pathogen detection in histological assays (Figure 3). To date, neither polyclonal nor monoclonal antibodies developed to *Perkinsus* sp. immunogens are parasite species-specific (Dungan and Roberson 1993, Romestand et al. 2001). Antibodies may be available from cited authors.

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- a. De-wax paraffin sections and hydrate to 0.1M phosphate-buffered saline (PBS), pH 7.2.
- b. Block sections with PBS + 0.05% (v/v) Tween-20 (PBST) + 2% (w/v) bovine serum albumin (BSA) + 1% normal serum.
- c. Incubate sections 30-60 min. in 5-20 $\mu\text{g/ml}$ primary antibody IgG diluted in PBST + 1% BSA (PBST/BSA).
- d. Wash sections in PBST.
- e. Incubate sections 30-60 min. in darkened, 1-4 $\mu\text{g/ml}$ affinity-purified secondary antibody/fluorochrome conjugate, diluted in PBST/BSA.
- f. Wash sections in PBST.
- g. Counterstain sections as desired with 0.01-0.05% Evan's blue or other appropriate fluorescent counterstain.
- h. Dip sections in PBST to rinse.
- i. Coverslip in pH 9-buffered glycerol, or equivalent mounting medium, and observe under fluorochrome-appropriate epifluorescence conditions.

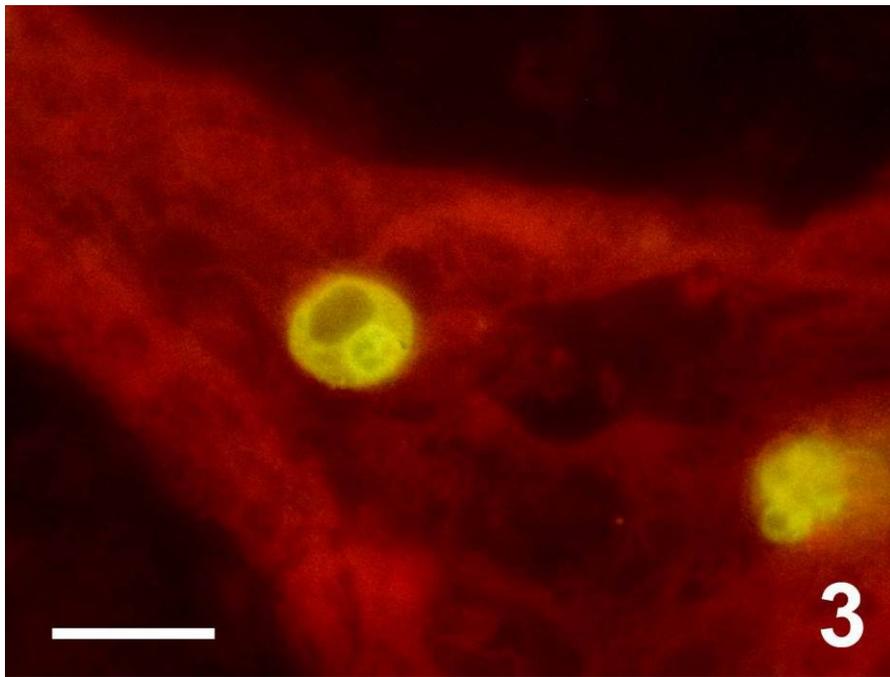


Figure 3. Fluorescence-immunostained *P. marinus* signet ring trophozoite in oyster digestive gland connective tissue. Antibodies label parasite membranes, nucleus, and cytoplasm, but not its eccentric vacuole. At lower right, a partly focused group of at least four smaller trophozoites reflect proliferative schizogony by a parent trophozoite. Bar = 10 μm . From Reece and Dungan (2006) with permission.

4. **ELISA assays**

Polyclonal antibody-based analyte-capture ELISA assays are reported to detect *P. marinus* extracellular secretions (ECP) in tissue homogenates from oysters with infection intensities of one parasite cell/g oyster tissue (Ottinger et al. 2001). The specificity of polyclonal antibodies against *P. marinus* ECP was not tested against other *Perkinsus* spp. or marine mollusc protistan associates.

Monoclonal murine IgG₁ antibodies against *P. marinus* in vitro trophozoites also label *P. olseni* cells, but do not recognize several other protistan oyster pathogens. A competitive ELISA assay using those antibodies is reported to detect 1,000 parasite cells in 50 µl of oyster tissue homogenate (Romestad et al. 2001).

***P. marinus* ECP-capture ELISA** (Ottinger et al. 2001)

- a. Coat ELISA plate wells with 50 µl of 40 µg/ml anti-*P. marinus* extracellular proteins (ECP) IgG in coating buffer for 1 h at 21 °C.
- b. Wash plate wells 3x with 0.1M TRIS-buffered saline (TBS, pH 7.2) + 0.1% (v/v) Tween-20 (TTBS).
- c. Block assay wells 1 h with 240 µl of TTBS + 1% (w/v) BSA (TTBS/BSA).
- d. Wash plate wells as above.
- e. Add 50 µl/well of oyster tissue homogenates or positive-control ECP samples, serially-diluted in TTBS/BSA as needed, and incubate for 2 h at 21 °C.
- f. Wash plate wells.
- g. Add 50 µl/well of 30 µg/ml biotinylated anti-*P. marinus*-ECP IgG in TTBS/BSA, for 1 h at 21 °C.
- h. Wash plate wells.
- i. Add 50 µl/well of streptavidin-horseradish peroxidase (HRPO) conjugate, diluted 1:1,000 in TTBS/BSA, for 1 h at 21 °C.
- j. Wash plate wells.
- k. Add 50 µl/well of ABTS chromogenic HRPO enzyme substrate; incubate reaction for 30 min in the dark at 21 °C.
- l. Read assay well absorbances (A_{450}), using ECP standards and negative control samples to interpret absorbances from diagnostic sample wells.

5. PCR assays

Numerous PCR assays are available to amplify genome target sequences unique to single *Perkinsus* species, to members of multiple species, or to members of the entire genus. Several well documented examples are listed in Table 2.

5a. *Perkinsus* genus PCR assay

Perkinsus genus-specific primers were designed (Casas et al. 2002) and refined (Audemard et al. 2004) to target the internal transcribed (ITS) regions of the ribosomal RNA gene complexes (rRNA) of all described *Perkinsus* species except *P. qugwadi (incertae sedis)* (Figure 4a). These primers amplify *Perkinsus* spp. DNAs isolated from cultured cells, as well as from DNA extracts of infected host tissue, and are able to detect less than 0.01 genome equivalents in a reaction (Figure 4b). Sequences of amplified DNA fragments may yield species identification through comparisons to GenBank-deposited ITS region sequences for *Perkinsus* species. (<http://www.ncbi.nlm.nih.gov/entrez/>)

- a. Extract genomic DNA from cultured cells or infected host tissue using one of many commercial DNA isolation kits, including the Qiagen DNeasy Blood & Tissue Kit™ (Qiagen), or the Quick-DNA Plus™ kit (Zymo Research). Alternatively, DNA can be extracted using phenol/chloroform-based (Reece et al. 1997) or CTAB-based DNA extraction methods (Carlini and Graves 1999).
- b. Quantify DNA spectrophotometrically.
- c. PCR amplification
 1. Primers: Perk ITS 85 (5'CCGCTTTGTTTRGMTCCC3')
 - Perk ITS 750 (5'ACATCAGGCCTTCTAATGATG3')
 2. Reagent concentrations for 25-µl reactions.
 - 10 ng (cultured cell) -100 ng (infected host tissue) of genomic DNA.
 - 20 mM Tris-HCl (pH 8.4)
 - 50 mM KCl, 1.5 mM MgCl₂
 - 1.5 mM MgCl₂
 - 0.2 mM each of dATP, dGTP, dCTP, dTTP
 - 25 pmol of each primer (above)
 - 0.625 units of *Taq* DNA polymerase
 - 0.2 mg/ml of BSA
 3. Cycling parameters (modified from Casas et al. 2002).
 - Initial denaturation of 4 min at 94 °C
 - 40 cycles of:
 - 30 sec at 94 °C
 - 30 sec at 55 °C
 - 1 min at 68 °C
 - Final extension of 5 min at 72 °C
- d. PCR products are electrophoresed in 1.5 - 2.0% agarose (in 1x TBE) gels, stained with ethidium bromide, and visualized using UV light. Expect amplicon sizes near 650 bp (Figure 4), with a known range of 638-670 bp.

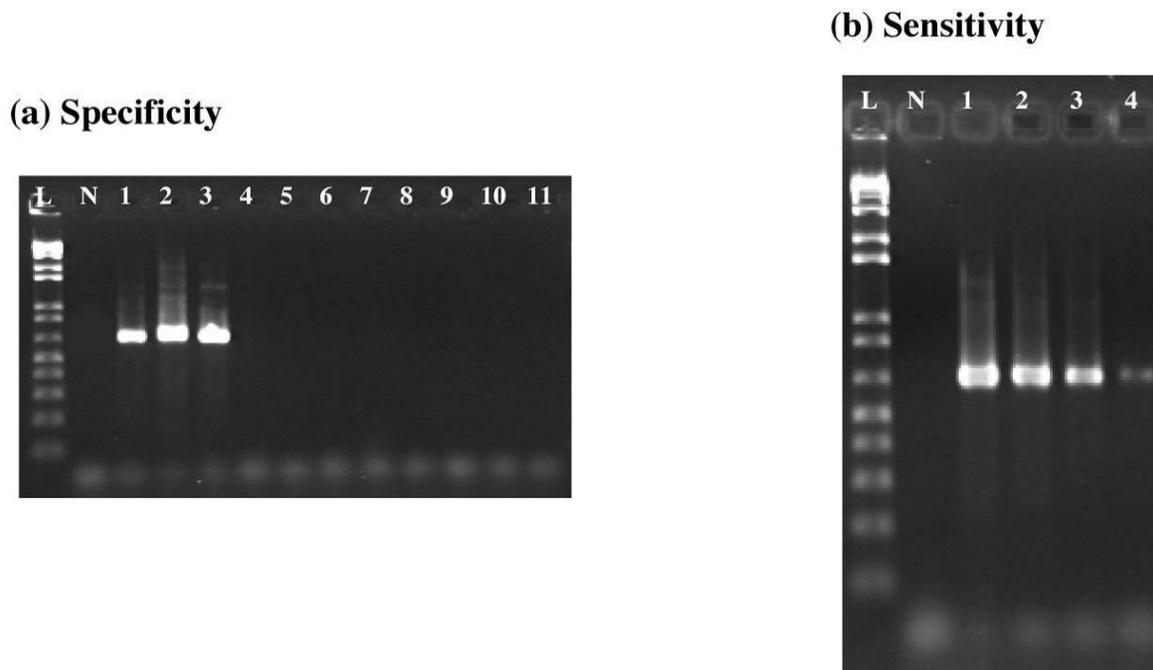


Figure 4. Ethidium bromide-stained PCR gels demonstrating the specificity and sensitivity of the *Perkinsus* genus-specific primer pair PerkITS-85 / PerkITS-750 (PerkITS). **(a)** Specificity of PerkITS primers tested on: no DNA (lane N), *P. marinus* (lane 1), *P. chesapeaki* (lane 2), *P. olseni* (lane 3), *Hematodinium perezii* (lane 4), *Pfiesteria piscicida* (lane 5), *Pseudopfiesteria schumwayae* (lane 6), *Amphidinium carterae* (lane 7), *Crypthecodinium cohnii* (lane 8), *Karlodinium micrum* (lane 9), *Peridinium foliaceum* (lane 10) and *Prorocentrum micans* (lane 11) DNAs. **(b)** Sensitivity of PerkITS primers tested on: no DNA (N), and serial dilutions of *P. marinus* DNA isolated from a determined (counted) number of cultured cells. DNA in each reaction was estimated to correspond with the following genome equivalents: 8 (lane 1), 0.8 (lane 2), 0.08 (lane 3), 0.008 (lane 4). Lanes L: 1 Kb Plus DNA Ladder (Invitrogen). Figure by C. Audemard; from Reece and Dungan (2006) with permission.

5b. Species-specific PCR

The *Perkinsus* species DNA amplicons from genus-specific PCR assays may be identified by either directly sequencing PCR products (Pagenkopp Lohan et al. 2018), or by sequencing cloned PCR products (Reece et al. 2008). Other methods of species identification include analyses of restriction fragment length polymorphisms (RFLP) of genus-specific amplicons (Abollo et al. 2006, Takahashi et al. 2009), or denaturing gradient gel electrophoresis (DGGE) of such amplicons (Ramilo et al. 2016).

Several species-specific PCR assays have been developed (Table 2). Most of these species-specific primers also target portions of the rRNA gene complex. Many assays are available to specifically detect *P. marinus* DNA (Marsh et al. 1995, Robledo et al. 1998, Yarnall et al. 2000, Audemard et al. 2004). To quantify parasites present in the host or the environment, Marsh et al. (1995) and Yarnall et al. (2000) developed semi-quantitative PCR assays; while Audemard et al. (2004) and Gauthier et al. (2006) developed real-time PCR assays (qPCR) for quantification of *P. marinus* or *Perkinsus* spp. DNAs in environmental samples. In addition, a multiplex PCR assay was developed to allow simultaneous and differential detection of *Haplosporidium nelsoni*, *H.*

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costale and *P. marinus*, all parasites of the eastern oyster *Crassostrea virginica* (Penna et al. 2001).

Species-specific PCR assays have also been developed for several other *Perkinsus* species. Nested ITS-region primers were developed to diagnose *P. olsenii* infecting Japanese Manila clams (Hamaguchi et al. 1998). Moss et al. (2006) developed a non-nested, *P. olsenii*-specific assay targeting the ITS region, which was refined (Reece et al. 2017). Several PCR primer pairs were designed to specifically target the non-transcribed spacer (NTS) region of *P. olsenii* (= *P. atlanticus*) (De la Herrán et al. 2000, Robledo et al. 2000, Murrell et al. 2002). Coss et al. (2001) developed a specific PCR assay targeting the NTS region of *P. chesapeaki* (= *P. andrewsi*) to discriminate this species from *P. marinus* and *P. olsenii*. Reece et al. (2017) developed another *P. chesapeaki*-specific assay that was tested against all currently described *Perkinsus* species. A *P. beihaiensis*-specific assay is also described (Moss et al. 2008).

Specific methods for *Perkinsus* species-specific PCR and qPCR assays are similar to those for the genus-specific assay. They are separately detailed in their cited references (Table 2).

Table 2. PCR primers and probes for genus- and species-specific standard and quantitative PCR for *Perkinsus* spp.

Target species	Target domain	Assay type	Primer/Probe name	Sequence (5' – 3')	Anneal temp. °C	Reference
<i>Perkinsus</i> genus	ITS	PCR	PerkITS-85F	CCGCTTTGTTTRGMTCCC	55-57	Casas et al. 2002 (modified)
			Perk ITS-750R	ACATCAGGCCTTCTAATGATG		
<i>Perkinsus</i> genus	Actin	PCR	PerkActin1-130F	ATGTATGTCCAGATYCAGGC	58.5	Moss et al. 2008
			PerkActin1-439R	CTCGTACGTTTTCTCCTTCTC		
<i>Perkinsus</i> genus	LSU	PCR	PerkITS2-217	GTGTTCCCTYGATCACGCGATT	58	Moss et al. 2008
			LSU-B	ACGAACGATTTGCACGTCAG		Lenears et al. 1989
<i>Perkinsus</i> genus	ITS	Taqman qPCR	PERK-f	TCCGTGAACCAGTAGAAATCTCAAC	60	Gauthier et al. 2006
			PERK-r	GGAAGAAGAGCGCACTGATATGTA		
			PERK-probe	CCCTTTGTGCAGTATGC		
<i>Perkinsus</i> genus	LSU	PCR	PerkITS2-217	GTGTTCCCTYGATCACGCGATT	58	Moss et al. 2008
			LSU-B	ACGAACGATTTGCACGTCAG		Lenears et al. 1989
<i>Perkinsus</i> genus	Actin	PCR	PerkActin1-130F	ATGTATGTCCAGATYCAGGC	58.5	Moss et al. 2008
			PerkActin1-439R	CTCGTACGTTTTCTCCTTCTC		
<i>P. beihaiensis</i>	ITS	PCR	PerkITS-85F	CCGCTTTGTTTRGMTCCC	57	Casas et al. 2002 (modified)
			PerkITS-430R	TCTGAGGGGCTACAATCAT		Moss et al. 2008
<i>P. chesapeaki</i>	ITS	PCR	PchesITS-5	CAAGAAGGACTGCGCTAG	59	Reece et al. 2017
			PchesITS-3	GTAACGATTCTGAGACCAAAGC		
<i>P. chesapeaki</i>	NTS	PCR	NTS-7	AAGTCGAATTGGAGGCGTGGTGAC	60	Coss et al. 2001
			NTS-6	ATTGTGTAACCACCCAGGC		
<i>P. honshuensis</i>	ITS	PCR	Phon-forward	AGACAGAGGCGGGCAGCAA	58	Kang et al. 2017
			Phon-reverse	AGACAGAGGCGGGCAGCAA		
<i>P. marinus</i>	ITS	PCR	Pmar-70F	CTTTTGYTWGAGWGTTGCGAGATG	57	Audemard et al. 2004
			Pmar-600R	CGAGTTTGCGAGTACCTCKAGAG		
<i>P. marinus</i>	ITS	Taqman qPCR	PMAR-f	TTGTTAACGCAACTCAATGCTTTGT	60	Gauthier et al. 2006
			PMAR-r	AAGCGCACATAACGAACCACC		
			PMAR-probe	GCTTGAACCTAACTCT		
<i>P. olseni</i>	ITS	PCR	PatI-140F	GACCGCCTTAACGGGCGGTGTT	64	Moss et al. 2006
			PatI-600R	GGRCTTGCGAGCATCCAAAG		

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6. SSU rRNA *in situ* hybridization assays (CISH, FISH)

The DNA probes for *Perkinsus* species *in situ* hybridization assays listed in Table 3 were specifically designed to be anti-sense. Therefore, they provide direct detection of abundant targets because they hybridize not only to rRNA genes in the cell nuclei, but also to their abundant cytoplasmic rRNA transcripts. A genus-*Perkinsus* probe that was developed to specifically target SSU rRNA sequences of *Perkinsus* species, was shown to hybridize specifically to all currently described *Perkinsus* sp. cells in paraffin-embedded histological sections of host species (Elston et al. 2004) (Figure 5). It has been tested, and it does not hybridize to *Perkinsus qugwadi (incertae sedis)*, to parasitic dinoflagellates, or to haplosporidian species. The *Perkinsus*-genus probe, Perksp700 (Table 3) may be 5' end-labeled with an antibody target ligand (ex. digoxigenin), or with a fluorochrome (ex. Alexa Fluor 488®).

Table 3. Genus- and species-specific ISH probes for *Perkinsus* spp. Target domains complementary to probe sequences occur in multi-copy genes for the listed genes (rDNA) and their transcribed rRNAs.

Target species	Target domain	Assay type	Probe name	Sequence (5' – 3')	Reference
<i>Perkinsus</i> genus	SSU	<i>In situ</i>	Perksp SSU-700	CGCACAGTTAAGTRCGTGRG CACG	Elston et al. 2004
<i>P. beihaiensis</i>	LSU	<i>In situ</i>	PerkBeh LSU	GTGAGTAGGCAGCAGAAGTC	Moss et al. 2008
<i>P. chesapeaki</i>	LSU	<i>In situ</i> cocktail	Pches LSU-485	CAGGAAACACCACGCACKAG	Reece et al. 2008
			Pches LSU-690	GCGAGCAATCTTAGAGCC	
<i>P. honshuensis</i> / <i>P. mediterraneus</i>	LSU	<i>In situ</i>	Pmed&Phon LSU-410	AGACAGAGGCGGGCAGCAA	Ramilo et al. 2015
<i>P. marinus</i>	LSU	<i>In situ</i> cocktail	Pmar LSU-181	GACAAACGGCGAACGACTC	Reece et al. 2008
			Pmar LSU-420	GAAGACAGGAGCGAGCAGC	
			Pmar LSU-560	AACCAATTCACAGATAGCG	
<i>P. olseni</i>	LSU	<i>In situ</i>	Pols LSU-689	ACTAAGTGCGGGCAATCTC	Reece et al. 2017

6a. Chromatic *in situ* hybridization assays (CISH) with digoxigenin-tagged probes are performed as previously described (Stokes and Bureson 1995, 2001, Marcino 2013), with the modifications included below. Similar CISH assays may use different reporter enzymes, such as horseradish peroxidase, with corresponding modifications to other reagents.

- a. Dewax paraffin-embedded tissue sections mounted on positively charged slides in xylene, and hydrate to 70% ethanol.

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- b. Wash slides in P buffer (50 mM Tris-HCl, pH 7.5; 5 mM EDTA) and treat with 125 µg/ml pronase solution in P buffer for 30 min. at 37 °C for permeabilization. Follow with two washes in P buffer and a 10-min incubation in 2x SSC (300 mM NaCl, 30 mM Na₃ citrate, pH 7.0).
- c. Prehybridize in a standard prehybridization solution for 1 h at 42 °C.
- d. Hybridize overnight in a humid chamber at 42 °C in a solution with probe concentrations of ~ 5-9 ng/µl in prehybridization buffer.
- e. Wash in a series of solutions descending from 2x SSC at 21 °C to 0.5x SSC at 42 °C.
- f. Chromogenic antibody detection of digoxigenin-labeled probes is done with an anti-digoxigenin antibody/alkaline phosphatase conjugate, after blocking and rinsing of hybridized sections. Assays are developed with a BCIP/NBT substrate, and Bismark brown or eosin counterstains may be included before coverslipping stained sections with compatible mounting media (Figure 5).

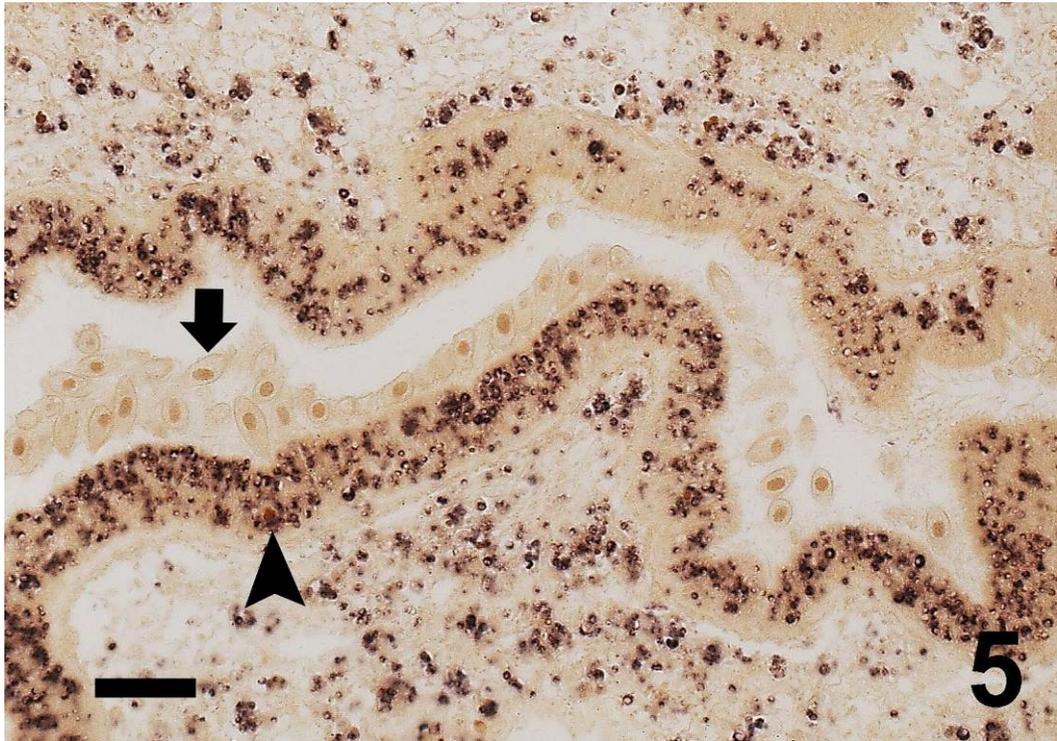


Figure 5. Chromogenic *in situ* hybridization (CISH) of genus *Perkinsus* SSU rRNA gene probe (violet-black) to *P. marinus* infecting intestine epithelium (arrowhead) and visceral connective tissues of *C. virginica*. Both parasite nuclear rDNA and cytoplasmic rRNA hybridize the digoxigenin-conjugated DNA probe, due to common sequence homologies. Polyploid macronuclei of large ciliates infesting the intestine lumen counterstain prominently with Bismark brown (arrow). Bar = 100 µm. From Reece and Dungan (2006) with permission.

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6b. Fluorescence *in situ* hybridization assays (FISH) with genus- or species-specificity for *Perkinsus* spp. are performed as previously described (Carnegie et al. 2006), and briefly listed below. Fluorochrome-labeled DNA probes provide direct detection of abundant targets, such as rRNA genes (rDNA) and their rRNA transcripts (Figure 6, Figure 7).

- Dewax, rehydrate, and permeabilize histological sections as described above for CISH assays.
- Wash slides for 10 min. in a 5% [v/v] acetic anhydride solution in 0.1M triethanolamine-HCl [pH 8.0]. (* optional)
- Rinse in PBS for 5 min.
- Incubate in 5x SET solution for 5 min (100 mM Tris-HCl, 6.4 mM EDTA, 750 mM NaCl, pH 8).
- Add probe to a concentration of ~5-9 ng/μl in prehybridization buffer and incubate overnight in a humid chamber at 42 °C.
- Wash with 0.2x SET (3 × 1 min at 42 °C).
- Coverslip with Fluoromount™ or other compatible mounting media.

* Acetylation of samples with acetic anhydride before hybridization, may reduce non-specific background signals.

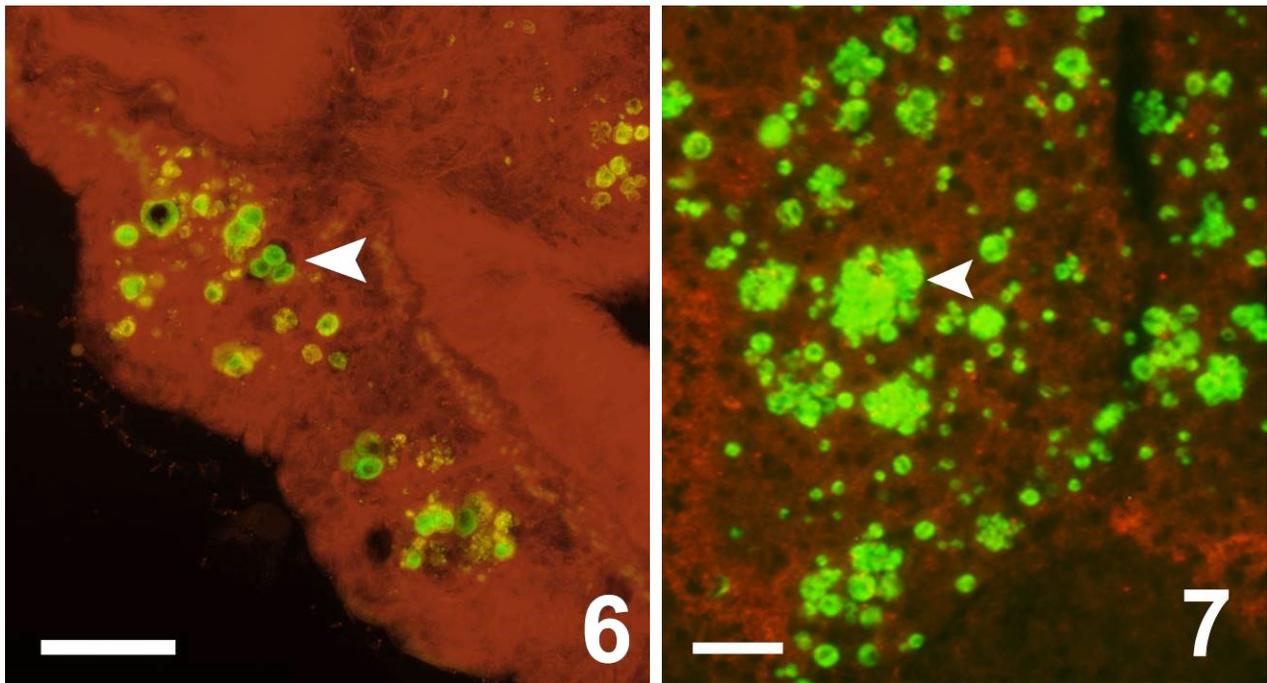


Figure 6-7. Fluorescence *in situ* hybridization (FISH) of probes to *Perkinsus* spp. in histological sections of infected molluscs. **6.** Vacuolated *Perkinsus chesapeaki* trophozoites (green) labeled by a species-specific LSU rRNA probe in a lesion within gill epithelium (arrowhead) and connective tissues of the clam *Mya arenaria*. Bar = 50 μm. **7.** Clusters of proliferating *Perkinsus marinus* trophozoites (green, arrowhead) labeled by a species-specific LSU rRNA probe in testicle tissues of the Brazilian oyster *Crassostrea gasar*. Bar = 20 μm.

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F. Procedures for Detecting Subclinical Infections

1. Histological immunoassays and *in situ* hybridization assays with DNA probes have visualized lesions with low parasite numbers in sections from oysters and clams (Ragone Calvo and Burreson 1994, Reece et al. 2008).
2. ELISA assays may detect infections of one parasite cell/g oyster tissue. (Ottinger et al. 2001).
3. RFTM whole body burden assays may detect one parasite cell/host. (Bushek et al. 1994).
4. PCR assay amplifications may detect subclinical infections when potentially rare parasite DNA target templates are present in amplified, 50-150 ng DNA sub-samples. Multiple-copy gene templates, such as rRNA gene targets, may permit detection of infections when <1 parasite genome complement is present in the amplified DNA sample. (Audemard et al. 2004, De Faveri et al. 2009).

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability or Preservation of the Etiological Agent

1. RFTM assays

- a. Ship or transport live molluscs humidified and cool (10-20 °C) to an analytical facility, or process aseptically where collected.
- b. Aseptically dissect and inoculate antimicrobial-fortified RFTM tubes, vials, or plate wells with mollusc tissue biopsies, to minimize contaminant overgrowth and cross-contamination of contents. Ship or transport inoculated culture vessels at 10-20 °C with expedience, for laboratory incubation and analysis.
- c. *In vitro Perkinsus* sp. cultures may be initiated in isohaline culture media, from aseptically collected tissues of infected hosts, or from *Perkinsus* sp. hypnospores enlarged in host tissue samples incubated in RFTM at 27 °C for 48-96 h (La Peyre and Faisal 1995, Dungan and Bushek 2015).

2. PCR assays

- a. Taking care to prevent sample cross-contamination, aseptically excise optimal host tissue biopsies for detection of parasite templates in extracted tissue DNAs. Alcohol-flame instruments and dissect each mollusc on a disposable, virgin plastic weigh boat or Petri dish. Sodium hypochlorite oxidation and hydrolysis of work surface and instrument contaminants between samples is an alternative decontamination method.
- b. Preserve tissue for subsequent DNA extraction by dehydration in ≥ 10 volumes of uncontaminated, 95-100% ethanol in sterile, virgin screw-capped containers. Store samples at or below 21 °C.

3. Histological assays

- a. Excise desired 5 mm-thick tissue samples, place in labeled histological cassettes, and fix immediately for 48 h in Davidson's or another fixative solution.
- b. After 24-48 h, decant fixative and replace with 70% ethanol. Infiltrate, embed, section, and stain tissue samples.

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