CHAPTER 2.4.6.

# INFECTION WITH *PERKINSUS OLSENI*

## 1. Scope

For the purpose of this chapter, infection with *Perkinsus olseni* is considered to be infection with *P. olseni*. *Perkinsus atlanticus* is considered to be a junior synonym.

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent, agent strains

The aetiological agent is *Perkinsus olseni*.

#### 2.1.2. Survival outside the host

Maximum survival time outside the host is unknown, but it is at least several months for prezoosporangia, or hypnospores (Casas *et al.,* 2002b).

#### 2.1.3. Stability of the agent (effective inactivation methods)

*Perkinsus olseni* is relatively stable because of its thick cell wall. Isolated *P. olseni* cells were killed by freshwater within 10 minutes at room temperature and by 0.006 mg $ml^{-1}$ = 6 ppm (parts per million) chlorine within 30 minutes (Goggin *et al.,* 1990). *Perkinsus olseni* cells in host tissue were much more resistant to these treatments. UV light (>28,000 µWs $cm^{-2}$) has been shown to inactivate *P. marinus* trophozoites (Bushek & Howell, 2000) and 60,000 µWs $cm^{-2}$ UV light has been shown to kill *P. olseni* hypnospores (Lester & Hayward, 2005).

#### 2.1.4. Life cycle

The life cycle is direct from host to host and all life stages are infective (Villalba *et al.,* 2004).

### 2.2. Host factors

#### 2.2.1. Susceptible host species

*Perkinsus olseni* has an extremely wide host range. Known hosts include the clams *Anadara trapezia*, *Austrovenus stutchburyi*, *Ruditapes decussatus*, *R. philippinarum, Tridacna maxima, T. crocea, Protothaca jedoensis* and *Pitar rostrata* (Goggin & Lester, 1995; Villalba *et al.,* 2004; Cremonte *et al.,* 2005; Park *et al.,* 2006; Sheppard & Phillips, 2008); oysters *Crassostrea gigas*, *C. ariakensis*, and *C. sikamea* (Villalba *et al.,* 2004); pearl oysters *Pinctada margaritifera, P. martensii*, and *P. fucata* (Goggin & Lester, 1995; Sanil *et al.,* 2010); abalone *Haliotis rubra, H. laevigata, H. scalaris,* and *H. cyclobates* (Goggin & Lester, 1995). Other bivalve and gastropod species might be susceptible to this parasite, especially in the known geographical range. Members of the families Arcidae, Malleidae, Isognomonidae, Chamidae and Veneridae are particularly susceptible, and their selective sampling may reveal the presence of *P. olseni* when only light infections occur in other families in the same habitat.

#### 2.2.2. Susceptible stages of the host

All stages after settlement are susceptible.

#### 2.2.3. Species or subpopulation predilection (probability of detection)

There is a wide host range and susceptibility varies (see 2.2.1 above); infection intensity increases with the age of the host.

#### 2.2.4. Target organs and infected tissue

Connective tissue of all organs, and haemocytes.

### 2.2.5. Persistent infection with lifelong carriers

Infection with *P. olseni* can be fatal depending on host and environmental conditions. Persistent infection with lifelong carriers can occur.

### 2.2.6. Vectors

None required as the life cycle is direct from host to host.

### 2.2.7. Known or suspected wild aquatic animal carriers

None known. Lester & Hayward (2005) examined 32 non-abalone mollusc species at Taylor Island, South Australia by Ray's fluid thioglycollate culture method (RFTM) and found no infections. Prevalence of *P. olseni* in abalone (*H. rubra*) at the site ranged from 26 to 56% in three different samples.

## 2.3. Disease pattern

### 2.3.1. Transmission mechanisms

Transmission is direct from host to host (Villalba *et al.,* 2004) and all life stages are infective.

### 2.3.2. Prevalence

Prevalence is highly variable depending on host and environmental conditions, but it is often 100%, as determined by histology or polymerase chain reaction (PCR). Prevalence is expected to be higher in individuals with more than 1 year of exposure to the pathogen.

### 2.3.3. Geographical distribution

Widespread throughout the tropical Pacific Ocean, Australia, North Island of New Zealand, Vietnam, Korea (Rep. of), Japan, China (People's Rep. of), Portugal, Spain, France, Italy, and Uruguay (Goggin & Lester, 1995; Villalba *et al.,* 2004; Cremonte *et al.,* 2005). Recently noted from India (Sanil *et al.,* 2010). The *Perkinsus* sp. reported from Thailand in the undulated surf clam *Paphia undulata* is almost certainly *P. olseni* based on DNA sequences from the parasite's internal transcribed spacer (ITS) regions. *Perkinsus olseni* is not known from North America.

### 2.3.4. Mortality and morbidity

Infections in clam hosts can be lethal depending on environmental conditions, and death may occur 1 or 2 years after infection. Infections in abalone in Australia appear not to cause mortality even though prevalence can be high (Lester & Hayward, 2005).

### 2.3.5. Environmental factors

The annual cycle of *P. olseni* is controlled by temperature. In *R. decussatus* in Spain, *P. olseni* infection intensity peaked in spring as temperature increased to about 15°C, remained high through the summer and early autumn when temperatures were 19–21°C, and then declined through winter and early spring coinciding with temperatures of 9–10°C (Villalba *et al.,* 2005). Highest host mortality occurred in early autumn during a period of maximum annual temperature. The salinity tolerance of *P. olseni* is poorly understood. Salinity remained above 15 practical salinity units (psu) during the study in Spain. Laboratory studies using *P. olseni* from culture (La Peyre *et al.,* 2006) suggest that *P. olseni* has optimum growth at 25 psu and is intolerant of salinity below 15 psu.

## 2.4. Control and prevention

### 2.4.1. Vaccination

None.

### 2.4.2. Chemotherapy

Cyclohexamide, pyrimethamine, deferoxamine (DFO) and 2, 2-bipyridyl inhibit *P. olseni in vitro*, and DFO inhibits *P. olseni in vivo*. (Elandalloussi *et al.,* 2005). N-Halamine disinfectant compounds are effective against cultured *P. marinus* cells in seawater (Delaney *et al.,* 2003), and bacitracin has been shown to reduce, but not eliminate *P. marinus* in infected oyster hosts (Faisal *et al.,* 1999). These compounds may be effective for *P. olseni* as well, but there has been no comparative study.

#### 2.4.3. Immunostimulation

None.

#### 2.4.4. Resistance breeding

None for *P. olseni*, although selective breeding has demonstrated some effectiveness for *P. marinus*.

#### 2.4.5. Restocking with resistant species

None.

#### 2.4.6. Blocking agents

None.

#### 2.4.7. Disinfection of eggs and larvae

*Perkinsus olseni* is not known to infect eggs or larvae of its hosts, but parasite cells may occur intercellularly. N-Halamine disinfectant compounds have shown effectiveness against *P. marinus* with no apparent effect on oyster larvae (Delaney *et al.,* 2003) and these compounds also may be useful for *P. olseni*.

#### 2.4.8. General husbandry practices

Low stocking density may reduce transmission of the pathogen.

## 3. Sampling

### 3.1. Selection of individual specimens

Live or freshly dead individuals should be sampled.

### 3.2. Preservation of samples for submission

For diagnosis using RFTM, samples must be fresh. For histology, the best preservative is Davidson's AFA, but 10% buffered formalin or other standard histology fixatives are also acceptable. For PCR assays, samples must be preserved in 95–100% ethanol and not denatured alcohol.

### 3.3. Pooling of samples

Pooling of very small spat (5–10 depending on size) is acceptable for PCR analyses.

### 3.4. Best organs or tissues

For RFTM with bivalves, pieces of gill, mantle and rectum are typically used. With abalone, tissue slices that include gill, mantle, and foot are cultured. For histology, a 5 mm thick section through the visceral mass that includes digestive gland, gill and mantle is used. For PCR, gill or mantle tissue is best.

### 3.5. Samples/tissues that are not suitable

Rectal tissue is not reliable for PCR assays because of the presence of inhibitors.

## 4. Diagnostic methods

### 4.1. Field diagnostic methods

#### 4.1.1. Clinical signs

Clinical signs are dead or gaping molluscs but these clinical signs are not specific to infection with *P. olseni*.

#### 4.1.2. Behavioural changes

Individual bivalves with late stage infections may be slow to close their valves, but these changes are not specific for *P. olseni.*

## 4.2. Clinical methods

### 4.2.1. Gross pathology

Gross signs are thin, watery tissue, pale digestive gland and nodules in mantle and gills of some hosts but these signs are not specific to infection with *P. olseni*.

### 4.2.2. Clinical chemistry

No data available.

### 4.2.3. Microscopic pathology

Fixed sections reveal large multifocal lesions in connective tissue containing *P. olseni* cells. Haemocyte infiltration (haemocytosis) occurs in most infections. In clam hosts, *P. olseni* cells are often encapsulated by a thick layer of eosinophilic material derived from haemocyte degranulation (Villalba *et al.,* 2004).

### 4.2.4. Wet mounts

Not recommended as a clinical method.

### 4.2.5. Smears

Not recommended as a clinical method.

### 4.2.6. Electron microscopy/cytopathology

Ultrastructural data show that the lysis of haemocytes and coalescence of metachromatic granules result in the nodule that encapsulates trophozoites (Sagrista *et al.,* 1995).

## 4.3. Agent detection and identification methods

### 4.3.1. Direct detection methods

#### 4.3.1.1. Microscopic methods

##### *4.3.1.1.1. Wet mounts*

Not recommended.

##### *4.3.1.1.2. Smears*

Smears are useful in advanced infections only.

*Samples to be taken:* live hosts.

*Technical procedure:* bleed host with a needle and syringe inserted into the adductor muscle of oysters and clams, or the cephalic sinus of abalone. Place a drop of haemolymph on a glass slide and smear. Observations are made at ×100–400 after Giemsa staining.

Positive/negative controls: none.

*Levels of validation:*

- Specificity and sensitivity: very low specificity with unknown sensitivity.
- Gold standard: sensitivity not validated against fluid thioglycollate culture (whole body burden assay [Fisher & Oliver, 1996]).

*Interpretation of results:*

- Presence of spherical, uninucleate cells 5–15 µm in diameter with a large vacuole and eccentric nucleus indicates the presence of *Perkinsus* sp. but the technique is not species specific.

*Availability of commercial tests:* quick staining kits are commercially available.

#### *4.3.1.1.3. Fixed sections*

*Samples to be taken:* live or freshly dead molluscs.

*Technical procedure:* sections of tissue that include digestive gland, gills and mantle should be fixed for 24 hours in Davidson's AFA or other standard histological fixative followed by normal processing for paraffin histology and staining with haematoxylin and eosin. Observations are made at increasing magnifications to ×400.

*Positive controls:* these are recommended and may be available from the OIE Reference Laboratory depending on the host.

*Levels of validation:*

- *Specificity and sensitivity:* species specificity is very low and sensitivity is good for moderate to heavy infections, but low for low intensity infections.
- *Gold standard:* fluid thioglycollate culture (whole body burden assay [Fisher & Oliver, 1996]) is the gold standard, although it is not species specific. Histology is not formally validated against fluid thioglycollate culture, although a recent study found histology to be less sensitive (Balseiro *et al*. 2010).

*Interpretation of results:*

- A positive result is the occurrence of spherical, uninucleate cells ranging from approximately 5 to 15 µm in diameter with a large vacuole and an eccentrically displaced nucleus with a prominent nucleolus. Multinucleate schizonts (dividing forms) often accompany the uninucleate trophozoites. Cells may be phagocytosed by host haemocytes Cells may be phagocytosed by host haemocytes. *Perkinsus olseni* cells stain lightly basophilic.
- In susceptible host species, in an area where only *P. olseni* is known to occur, a positive result is presumptive evidence of *P. olseni* infection, but should be confirmed by *in-situ* hybridisation (ISH) or DNA sequencing because of the possible presence of other *Perkinsus* species.

*Availability of commercial tests:* no commercially available tests.

#### *4.3.1.1.4. Ray's fluid thioglycollate culture method (RFTM)*

Incubation in thioglycollate is routinely used for surveillance of *P. olseni.* The technique is simple, inexpensive and very sensitive, but not species specific. Trophozoites of *P. olseni* in host tissue will enlarge when cultured for at least 5 days in fluid thioglycollate medium containing dextrose that is fortified with antibiotics (penicillin, streptomycin) and an antifungal compound (nystatin) to reduce bacterial and fungal growth. When the tissue is macerated after culture to allow penetration of aqueous iodine solution (Lugol's), the enlarged trophozoites (hypnospores or prezoosporangia in the old terminology) readily take up Lugol's and they easily become visible at low power because of their generally bluish–black coloration and their spherical shape.

*Samples to be taken:* live or freshly dead molluscs.

*Technical procedure:*

Tissue assay (Ray, 1966): tissue samples measuring approximately 5–10 mm are excised giving preference to rectal, gill and mantle tissue from oysters and clams, and adductor or foot muscles or mantle for abalone, and placed in test tubes containing thioglycollate medium (thioglycollate medium containing dextrose 14.6 g; NaCl, 10.0 g; sterile distilled water ($dH_2O$), 485 ml). A total of 9.5 ml is dispensed into disposable test tubes, which are autoclaved for 15 minutes at 1.2 kg $cm^{-2}$ pressure. The autoclaved solution can be stored in tubes for up to 3 weeks. Dissecting utensils should be rinsed in 95% ethanol and flamed between hosts to prevent carry-over. The recommended antifungal/antibiotics are: 500 units $ml^{-1}$ penicillin G and 500 units $ml^{-1}$ dihydro-streptomycin in media (penicillin, 3.13 g; streptomycin, 6.55 g; 500 ml $dH_2O$; freeze in 50 ml aliquots; add 0.5 ml to each tube), and 50 µl of mycostatin (nystatin) per tube. Chloromycetin can be used in place of penicillin/streptomycin. The tube is plugged with a foam rubber or cotton stopper. Incubation is at 22–25°C for between 5 and 7 days, in the dark. After incubation, the fragments of tissue are collected and chopped with a scalpel blade on a glass slide, a drop of Lugol's iodine solution is added (stock Lugol's iodine solution: potassium iodide, 6.0 g; iodine, 4.0 g; $dH_2O$, 100 ml. Lugol's iodine working solution: $dH_2O$, 30.0 ml; Lugol's stock solution, 15.0 ml) and the preparation is covered with a cover-slip and allowed to sit for 10 minutes. The preparations are examined in the fresh state.

Whole body burden assay (Fisher & Oliver, 1996): the entire host, cut into 2–5 mm pieces, is placed in fluid thioglycollate culture medium and incubated as in the tissue assay above. If host organisms are too large to use the entire host, then selected target tissue can be used. The solution is centrifuged at 1500 ***g*** for 10 minutes and the supernatant is discarded. 2 M NaOH (20 ml $g^{-1}$ tissue) is added and the solution is incubated at 60°C for 2–6 hours until tissue is digested. The solution is centrifuged at 1500 ***g*** for 10 minutes and the supernatant is discarded. The solution is washed three times in deionised water, the pellet is resuspended in 1 ml Lugol's iodine working solution, and the cells are counted. Serial dilutions may have to be made to reduce the total cell number to a manageable number.

*Levels of validation:*

- *Specificity and sensitivity:* specificity is low as the technique does not distinguish between species of *Perkinsus*. Sensitivity is high, especially for the whole body burden assay (Bushek *et al.,* 1994).
- *Gold standard:* fluid thioglycollate culture (tissue assay) is the recommended surveillance method. The tissue assay has not been validated against the whole body burden assay for *P. olseni,* but for *P. marinus* the tissue assay has been shown to be less sensitive (Bushek *et al.,* 1994). A recent study comparing the RFTM tissue assay to histology and a newly designed PCR assay found the RFTM assay to be the most sensitive of the three (Balseiro *et al*., 2010).

*Interpretation of results:*

- Cultured parasites enlarge from 5–15 to 50–70 µm during incubation. *Perkinsus* spp. cells are spherical and the walls stain generally blue or bluish-black with Lugol's iodine solution (Ray, 1966).
- In susceptible host species, within the known range of *P. olseni*, a positive result is presumptive evidence of *P. olseni* infection, but should be confirmed by species-specific PCR, *in-situ* hybridisation (ISH) and/or DNA sequencing of the ITS (internal transcribed spacer) region because of the possible presence of other *Perkinsus* species. If no parallel samples are preserved for molecular diagnostics, parasite DNA may be extracted and PCR-amplified directly from positive thioglycollate preparations (Audemard *et al.,* 2008).

*Availability of commercial tests:* no commercial kits available.

#### 4.3.1.2. Agent isolation and identification

##### *4.3.1.2.1. Cell culture/artificial media*

*Perkinsus* spp. cells are easily cultured in a variety of media (Casas *et al.,* 2002a; Dungan & Hamilton, 1995; La Peyre *et al.,* 1993). Culture medium is usually inoculated with heart, haemolymph, or gill tissue. Comparisons of commercially available media for the culture of *P. marinus,* but not *P. olseni,* have been made (Dungan & Hamilton, 1995). The *P. marinus* isolate used proliferated most rapidly in 1:1 DME/Ham's F-12 medium.

##### *4.3.1.2.2. Antibody-based antigen detection methods*

Polyclonal antibodies have been developed for a cell wall component of *P. olseni* (Montes *et al.,* 2002), but they also bind to *P. marinus*. No diagnostic assay has been developed using these antibodies.

##### *4.3.1.2.3. Molecular techniques*

Perkinsus *genus assays* (*PCR and* in-situ *hybridisation*)

For surveillance using PCR, it is recommended that *Perkinsus* genus PCR assays be conducted first, and then samples with positive results should be tested with a *P. olseni*-specific assay. Much more is known about inter- and intra-specific sequence variation of the ITS region than the non-transcribed spacer (NTS) region of the *Perkinsus* sp. rRNA gene complex, based on sequences available in the National (USA) Center for Biotechnology Information GenBank database. Therefore, PCR primers that target the ITS region are recommended as one can be more confident that they will detect various *P. olseni* strains. For *in-situ* hybridisation (ISH), probes have been developed that target the small subunit (SSU) of the rRNA gene complex (Elston *et al.,* 2004). In addition, a real-time *Perkinsus* genus PCR assay has been developed for use with host tissue (Gauthier *et al.,* 2006). It has been tested only with *P. marinus, P. olseni* and *P. chesapeaki,* and was shown to be more sensitive in a limited validation against the RFTM assay. This assay needs

to be tested more thoroughly for specificity, but may be useful for laboratories that possess the necessary equipment.

#### *4.3.1.2.3.1.* Perkinsus *genus-specific polymerase chain reaction*

*Samples to be taken:* live or freshly dead molluscs. 2–3 $mm^2$ tissue pieces are excised aseptically from gill and mantle and placed into 1.5 ml microcentrifuge tubes containing 95–100% ethanol. Dissecting utensils should be flamed between samples to prevent cross-contamination.

*Technical procedure:* DNA is extracted by proteinase K digestion overnight at 56°C and the spin-column methodology using commercially available kits. Recommended *Perkinsus* genus-specific PCR primers are those of Audemard *et al.,* (2004). The forward primer PerkITS-85 (5'-CCG-CTT-TGT-TTG-GAT-CCC-3') and reverse primer PerkITS-750 (5'-ACA-TCA-GGC-CTT-CTA-ATG-ATG-3') target the ITS region of the rRNA gene complex. These primers amplify a 703 bp product and can be used to detect DNA from any known and possibly unknown species of *Perkinsus*, except *P. qugwadi*. Each PCR reaction contains the following: 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, each primer at 0.1 µM, 0.025 U $µl^{-1}$ *Taq* polymerase, 0.05 mg $ml^{-1}$ BSA (bovine serum albumin), and 0.5 µl genomic DNA (10–50 ng total). Amplification conditions are an initial denaturation at 95°C for 4 minutes followed by 40 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, with a final elongation at 72°C for 10 minutes. Following amplification, 4 µl of PCR product is visualised on a 2% agarose gel.

*Positive/negative controls:* these are compulsory. The positive control is genomic DNA from any *Perkinsus* sp.-infected mollusc (except *P. qugwadi*). Negative controls are either no-DNA assays or assays with uninfected molluscs.

*Levels of validation:*

- *Specificity and sensitivity:* the *Perkinsus* genus PCR primers have been tested for inclusivity against all known *Perkinsus* species, and tested for specificity against a variety of haplosporidians, and parasitic and non-parasitic dinoflagellates (Reece & Dungan, 2005). Sensitivity has not been compared with the RFTM.

*Interpretation of results:*

- A positive result is the presence of a band of the appropriate size (703 bp) in an agarose gel, with all negative controls negative and all positive controls positive.

#### *4.3.1.2.3.2.* Perkinsus *genus-specific* in-situ *hybridisation*

*Samples to be taken:* follow the procedure for 'fixed sections' (4.3.1.1.3) above, except that tissue sections must be placed on positively charged glass slides or slides coated with 3-aminopropyl-triethoxylane, without staining. Deparaffinise sections in xylene for 10 minutes and then rehydrate in an alcohol series. Wash sections twice for 5 minutes in phosphate-buffered saline (PBS).

*Technical procedure:* a specific DNA probe that targets the small subunit rRNA gene has been developed for the genus *Perkinsus* (Elston *et al.,* 2004): Perksp700DIG (5'-CGC-ACA-GTT-AAG-TRC-GTG-RGC-ACG-3'). The probe should be 5' end-labelled with digoxigenin.

The tissue sections are treated with 125 µg $ml^{-1}$ pronase in PBS, at 37°C for 30 minutes. The reaction is then stopped by washing the sections in PBS with 0.2% glycine for 5 minutes. The sections are then placed in 2× SSC (standard saline citrate; 20× SSC = 3 M NaCl; 0.3 M Na-citrate; pH 7.0) for 10 minutes.

The sections are prehybridised for 1 hour at 42°C in prehybridisation solution (4× SSC, 50% formamide, 5× Denhardt's solution, 0.5 mg $ml^{-1}$ yeast tRNA, and 0.5 mg $ml^{-1}$ heat-denatured salmon sperm DNA) in a humid chamber.

The prehybridisation solution is then replaced with prehybridisation buffer containing 7 ng $µl^{-1}$ of the digoxigenin-labelled *Perkinsus* genus probe. The sections are covered with *in-situ* hybridisation plastic cover-slips and placed on a heating block at 90°C for 12 minutes. The slides are then cooled on ice for 1 minute before hybridisation overnight at 42°C in a humid chamber.

The sections are washed twice for 5 minutes each in 2× SSC at room temperature, twice for 5 minutes each in 1× SSC at room temperature, and twice for 10 minutes each in 0.5× SSC at 42°C. The sections are then placed in Buffer 1 (100 mM Tris, pH 7.5, 150 mM NaCl) for 1–2 minutes.

The sections are placed in Buffer 1 (see above) supplemented with 0.3% Triton X-100 and 2% sheep serum for 30 minutes. Anti-digoxigenin alkaline phosphatase antibody conjugate is diluted 1/500 (or according to the manufacturer's recommendations) in Buffer 1 supplemented with 0.3% Triton X-100 and 1% sheep serum and applied to the tissue sections. The sections are covered with *in-situ* hybridisation cover-slips and incubated for 3 hours at room temperature in a humid chamber.

The slides are washed twice in Buffer 1 for 5 minutes each and twice in Buffer 2 (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 5 minutes each. The slides are then placed in colour development solution (337.5 µg $ml^{-1}$ nitroblue tetrazolium, 175 µg $ml^{-1}$ 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 240 µg $ml^{-1}$ levamisole in Buffer 2) for 2 hours in the dark. The colour reaction is stopped by washing in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA [ethylene diamine tetra-acetic acid]).

The slides are then rinsed in sterile distilled water ($dH_2O$). The sections are counterstained with Bismarck Brown Y, rinsed in $dH_2O$, and cover-slips are applied using an aqueous mounting medium.

*Positive/negative controls:* these are compulsory. Positive controls are tissue sections from any *Perkinsus* sp.-infected mollusc. Negative controls are either no-probe assays or assays with uninfected oysters.

*Levels of validation:*

- *Specificity and sensitivity:* the *Perkinsus* genus DNA probe has been tested for specificity against a variety of *Perkinsus* species, haplosporidians, and parasitic dinoflagellates (Elston *et al.,* 2004). Sensitivity is greater than paraffin histology but the probe has not been compared with the RFTM.

*Interpretation of results:*

- A positive result is the presence of purple-black labelling of the parasite cells, with all negative controls negative and all positive controls positive.

### Perkinsus olseni *assays* (*PCR and* in-situ *hybridisation*)

#### *4.3.1.2.3.* Perkinsus olseni-*specific polymerase chain reaction*

PCR primers that target the NTS region and the ITS region of the rRNA gene complex have been developed for *P. olseni.* Although the primers that target the NTS region have demonstrated good species specificity, little is known of the variation within species for the NTS region and there is a risk of false negatives. The sequence variation in the ITS region is more broadly characterised (see the GenBank database) and primers targeting the ITS region are more thoroughly tested for specificity. For these reasons, primers that target the ITS region are recommended. The most recent version of the recommended specific *P. olseni* ITS assay is presented here. It is recommended that surveillance using the *Perkinsus* genus ITS assay be conducted first, and then the specific assay. A PCR-restriction fragment length polymorphism (RFLP) assay has been developed that may be useful for specific diagnoses of *P. olseni* (Abollo *et al.,* 2006), although it has not been tested for specificity against all known *Perkinsus* species.

*Samples to be taken:* live or freshly dead molluscs. 2–3 $mm^2$ tissue pieces are excised aseptically from gill and mantle and placed into 1.5 ml microcentrifuge tubes containing 95–100% ethanol. Dissecting utensils should be flamed between samples to prevent cross-contamination.

*Technical procedures:* DNA is extracted by proteinase K digestion overnight at 56°C and the spin-column methodology using commercially available kits. PCR primers that target the ITS region of the *P. olseni* rRNA gene complex have been developed (Moss *et al.,* 2006): forward primer PolsITS-140F (5'-GAC-CGC-CTT-AAC-GGG-CCG-TGT-T-3') and reverse primer PolsITS-600R (5'-GGR-CTT-GCG-AGC-ATC-CAA-AG-3'. The amplified product size is approximately 450 bp. The PCR reaction mixtures contain PCR buffer at a concentration of 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, each primer at 0.1 µM, 0.025 U $µl^{-1}$ *Taq* polymerase, 0.0 mg $ml^{-1}$ BSA and 0.5 µl genomic DNA (~10–50 ng) in a total volume of 25 µl. Amplifications are performed with an initial denaturation of 95°C for 4 minutes followed by 40 cycles of: 94°C for 1 minute, 62°C for 1 minute, 65°C for 3 minutes, with a final elongation step of 65°C for 10 minutes.

PCR products are eletrophoresed on 2% agarose (in 1× TAE or TBE) gels, stained with ethidium bromide, and visualised using UV light.

*Positive/negative controls:* these are compulsory. Positive controls are DNA from purified *P. olseni* cells, or genomic DNA from heavily infected hosts. Negative controls are no target DNA reactions.

*Levels of validation:*

- *Specificity and sensitivity:* ITS region primers have been tested for specificity against *P. marinus*, *P. chesapeaki, P. mediterraneus* and *P. honshuensis* (Moss, 2007; Moss *et al.,* 2006). Sensitivity is high with the ability to detect one *P. olseni* cell in 30 mg of oyster tissue, but subsampling error in light, localised infections may lead to false negatives.
- *Gold standard:* the ITS PCR assay for *P. olseni* has been validated to a limited extent against RFTM (tissue assay) and shown to be more sensitive (Moss, 2007).

*Interpretation of results:*

- A positive result is a PCR amplification product of the appropriate size (455 bp), with all negative controls negative and all positive controls positive.

*Availability of commercial tests:* not commercially available.

#### *4.3.1.2.3.4.* Perkinsus olseni-*specific* in-situ *hybridisation*

*Samples to be taken:* follow procedure for 'fixed sections' (4.3.1.1.3) above, except that tissue sections must be placed on positively charged glass slides or slides coated with 3-aminopropyl-triethoxylane, without staining. Deparaffinise sections in xylene for 10 minutes and then rehydrate in an alcohol series. Wash sections twice for 5 minutes in PBS.

*Technical procedures:* a DNA probe that targets the LSU of the rRNA gene of *P. olseni* has been developed (Moss *et al.,* 2006) (PolsLSU-464DIG 5'-CTC-ACA-AGT-GCC-AAA-CAA-CTG-3'). The probe should be end-labelled with digoxygenin. The ISH procedures are the same as for the *Perkinsus* genus probe presented above.

*Positive/negative controls:* these are compulsory. Positive controls are tissue sections from any susceptible host infected with *P. olseni*. Negative controls are either no-probe assays or assays with uninfected oysters.

*Levels of validation:*

- *Specificity and sensitivity:* the *P. olseni* DNA probe has been tested for specificity against a variety of *Perkinsus* species (Moss, 2007; Moss *et al.,* 2006), including *P. marinus, P. chesapeaki, P. mediterraneus*, and *P. honshuensis*. Sensitivity is greater than paraffin histology but the probe has not been compared with the RFTM.

*Interpretation of results:*

- A positive result is the presence of purple-black labelling of the parasite cells, with all negative controls negative and all positive controls positive.

#### *4.3.1.2.4. Agent purification*

*Perkinsus olseni* can be purified by development of clonal cultures.

### 4.3.2. Serological methods

None applicable.

## 5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of *Perkinsus olseni* are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

***Table 5.1.*** *Methods for targeted surveillance and diagnosis*

| Method | Targeted surveillance | | | Presumptive diagnosis | Confirmatory diagnosis |
|---|---|---|---|---|---|
| | Seed | Juveniles | Adults | | |
| **Gross signs** | d | d | d | d | d |
| **Haemolymph smears** | d | c | c | c | d |
| **Histopathology** | b | b | b | b | d |
| **RFTM, tissue assay*** | d | a | a | b | d |
| **RFTM, body burden assay*** | d | c | c | c | d |
| **PCR** | a | b | b | a[1] | b[1] |
| ***In-situ* DNA probes** | d | b | b | b | a |
| **Sequence** | d | d | d | d | b[1] |

RFTM = Ray's fluid thioglycollate culture method; *the technique is not species specific, but can be used reliably in hosts/areas where only one species of *Perkinsus* is present or predominant; [1]should be used only if infection is visualised by smears, RFTM or histology; PCR = polymerase chain reaction.

## 6. Test(s) recommended for targeted surveillance to declare freedom from infection with *Perkinsus olseni*

PCR assays in combination with RFTM tissue assays or, if possible, whole body burden assays should be used for targeted surveillance to declare freedom from infection with *P. olseni*.

## 7. Corroborative diagnostic criteria

### 7.1. Definition of suspect case

In known susceptible species within the known geographical range of *P. olseni*, a suspect case of infection with *P. olseni* is a positive result by any one of the following methods: haemolymph smear, histology, fluid thioglycollate culture or PCR. In other host species, or outside the known range of *P. olseni*, a suspect case is a positive result by PCR. Such cases should be submitted to the OIE Reference Laboratory for confirmation.

### 7.2. Definition of confirmed case

A confirmed case of *P. olseni* is a positive result by haemolymph smear, histology or fluid thioglycollate culture combined with a positive result with PCR or ISH. Sequencing of the ITS region is recommended as a final step for a confirmatory diagnosis.

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**NB:** Currently (2021) there is no OIE Reference Laboratory for infection with *Perkinsus olseni* (see Table at the end of this *Aquatic Manual* or consult the OIE web site: https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

**NB:** FIRST ADOPTED IN 1995 AS PERKINSOSIS. MOST RECENT UPDATES ADOPTED IN 2015.