CHAPTER 2.4.5.

INFECTION WITH *PERKINSUS MARINUS*

1. Scope

For the purpose of this chapter, infection with *Perkinsus marinus* is considered to be infection with *P. marinus*, the causative agent of dermo disease in oysters.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agent is represented by all strains of *Perkinsus marinus*.

2.1.2. Survival outside the host

Maximum survival time outside the host is unknown.

2.1.3. Stability of the agent (effective inactivation methods)

Perkinsus marinus is relatively stable because of its thick cell wall. Desiccation, chlorination (>0.3 mg ml^{-1} = 300 ppm [parts per million]), UV light (>28,000 µWs cm^{-2}) and freshwater have all been shown to inactivate *P. marinus* cells (Bushek *et al.,* 1997; Bushek & Howell 2000). UV irradiation from 4000 to 14,000 µWs cm^{-2} will inhibit proliferation of *P. marinus* (Bushek & Howell, 2000).

2.1.4. Life cycle

The life cycle is direct from host to host and all life stages are infective (Andrews, 1996; Villalba *et al.,* 2004).

2.2. Host factors

2.2.1. Susceptible host species

Eastern oyster, *Crassostrea virginica*; Pacific oyster, *C. gigas*; suminoe oyster, *C. ariakensis;* mangrove oyster, *C. rhizophorae*; Cortez oyster, *C. corteziensis* (Andrews 1996; Calvo *et al.,* 1999; Calvo *et al.,* 2001; Villalba *et al.,* 2004; Cáceres-Martínez *et al.,* 2008); softshell clam, *Mya arenaria*; Baltic macoma, *Macoma balthica* (Dungan *et al.,* 2007).

2.2.2. Susceptible stages of the host

All stages after settlement are susceptible.

2.2.3. Species or subpopulation predilection (probability of detection)

Crassostrea virginica is the most susceptible species; *C. gigas* and *C. ariakensis* can be infected, but infections are usually light (Calvo *et al.,* 1999; 2001). Prevalence in the clams *M. arenaria* and *M. balthica* in nature is less than 10% (Reece *et al.,* 2008). *Perkinsus marinus* infection of *C. corteziensis* requires more study, but this host appears to be intermediate in susceptibility between *C. virginica* and Asian species *C. gigas* and *C. ariakensis* (Cáceres-Martínez *et al.,* 2008; Dungan *et al.,* 2007).

2.2.4. Target organs and infected tissue

Gut epithelium, connective tissue of all organs, and haemocytes (Mackin, 1951).

2.2.5. Persistent infection with lifelong carriers

Infection with *P. marinus* is usually fatal depending on host and environmental conditions (Andrews, 1996; Burreson & Ragone Calvo, 1996). Persistent infection with lifelong carriers can occur.

2.2.6. Vectors

No vectors are required: the life cycle is direct.

2.2.7. Known or suspected wild aquatic animal carriers

None known.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Transmission is direct from host to host. All life stages are infective (Villalba *et al.,* 2004). Viable cells are released in host faeces (Bushek *et al.,* 2002b) or upon death of the host, and are acquired through host feeding mechanisms.

2.3.2. Prevalence

Prevalence is highly variable depending on salinity and host factors, but it is often 100% in *C. virginica*. Prevalence is expected to be higher in individuals with more than 1 year of exposure to the pathogen (Andrews, 1996; Burreson & Ragone Calvo, 1996). Prevalence in clams is low, usually less than 10%.

2.3.3. Geographical distribution

East coast of North America from Maine, USA to Campeche, Mexico. Recently introduced to the Pacific coast of Mexico (Cáceres-Martínez *et al.,* 2008).

2.3.4. Mortality and morbidity

Infection is often lethal for *C. virginica*. Death usually occurs 1 or 2 years after infection, during or shortly after the warmest annual water temperatures (Burreson & Ragone Calvo, 1996). Infection intensity in clams is very low and there is no indication of mortality.

2.3.5. Environmental factors

Prevalence and intensity of *P. marinus* infections are greatest at salinities greater than 12 practical salinity units (psu). Transmission can occur between 9 and 12 psu, but infections remain low in intensity. *Perkinsus marinus* can persist for long periods in hosts at salinities less than 9 psu, but replication is low and no host mortality occurs. Temperature controls the annual cycle of *P. marinus*, with maximum prevalence and intensity lagging 1–2 months behind maximum summer water temperatures and minimum prevalence and intensity lagging 1–2 months behind minimum winter temperatures. Thus, *P. marinus* infections are most intense in autumn and least intense in early spring (Burreson & Ragone Calvo, 1996).

2.4. Control and prevention

2.4.1. Vaccination

None.

2.4.2. Chemotherapy

N-Halamine disinfectant compounds killed cultured *P. marinus* cells without affecting oyster larvae (Delaney *et al.,* 2003). Bacitracin, cycloheximide and freshwater have been shown to reduce, but not eliminate *P. marinus* in infected oyster hosts (Calvo & Burreson, 1994; Faisal *et al.,* 1999; La Peyre *et al.,* 2003). These treatments may be relevant for aquaculture, but are not practical in the natural environment.

2.4.3. Immunostimulation

None.

2.4.4. Resistance breeding

Selective breeding of surviving oysters from epizootics has demonstrated effectiveness for reducing mortality caused by *P. marinus* (Ragone Calvo *et al.,* 2003).

2.4.5. Restocking with resistant species

Selectively-bred disease-tolerant strains of *C. virginica* are used in aquaculture in Chesapeake Bay and elsewhere (Ragone Calvo *et al.,* 2003), but are not recommended for restoration of natural oyster stocks because of genetic issues.

2.4.6. Blocking agents

None.

2.4.7. Disinfection of eggs and larvae

Perkinsus marinus is not known to infect eggs or larvae, but cells could occur extracellulary. Disinfection of eggs or larvae may be possible using N-halamine disinfectant compounds (Delaney *et al.,* 2003).

2.4.8. General husbandry practices

Farming in areas where salinity is less than 12 psu and use of fast-growing, disease-tolerant strains has shown some benefit (Andrews, 1996; Burreson & Ragone Calvo, 1996).

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 the Ray's fluid thioglycollate culture method (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 polymerase chain reaction (PCR) assays, samples must be preserved in 95–100% ethanol and not denatured alcohol.

3.3. Pooling of samples

Pooling of samples from juvenile and adults is not advisable, as it would reduce the mass of tissue evaluated per individual and thus the sensitivity of detection. Pooling of very small spat (five to ten individuals depending on size) or larvae is acceptable for whole-body PCR analyses, although this makes it impossible to estimate pathogen prevalence accurately.

3.4. Best organs or tissues

For the RFTM, pieces of gill, mantle and rectum are typically used. 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

Perkinsus marinus causes a chronic wasting disease. Clinical signs may be dead or gaping bivalves with thin, watery tissue but these clinical signs are not specific to infection with *P. marinus*.

4.1.2. Behavioural changes

Infected hosts may be slow to close their valves when disturbed but these behavioural changes are not specific to infection with *P. marinus*.

4.2. Clinical methods

4.2.1. Gross pathology

Gross signs are thin, watery tissue but these gross signs are not specific to infection with *P. marinus*.

4.2.2. Clinical chemistry

None.

4.2.3. Microscopic pathology

Fixed sections reveal large multifocal lesions in gut epithelium or connective tissue of any organ containing *P. marinus* cells (Mackin, 1951). Haemocyte infiltration and phagocytosis of *P. marinus* cells occurs in most infections. In high intensity infections, the gut epithelium may be almost completely destroyed.

4.2.4. Wet mounts

Not recommended as a clinical method.

4.2.5. Smears

Not recommended as a clinical method.

4.2.7. Electron microscopy/cytopathology

No data available.

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. Place a drop of haemolymph on a glass slide and smear. Observations are made at ×100–400 after 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 cells 2–15 µm in diameter with a large vacuole and eccentric nucleus, often phagocytosed by host haemocytes, 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 oysters.

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

Positive controls: these are recommended and are available from the OIE Reference Laboratory.

Levels of validation:

- *Specificity and sensitivity:* species specificity is very low, and sensitivity is good for moderate- to high-intensity infections, but low for low-intensity infections.
- *Gold standard:* fluid thioglycollate culture (whole body burden assay) is the gold standard although it is not species specific. Histology is less sensitive, but not formally validated against fluid thioglycollate culture.

Interpretation of results:

- A positive result is the occurrence of spherical, uninucleate cells ranging from about 2 to 5 µm (sometimes to 10 µm) in diameter with a large vacuole and an eccentrically displaced nucleus. These are typically associated with host gut or sometimes epithelium in lighter infections, with colonisation of connective tissues characteristic of more advanced cases. Binucleate *P. marinus* schizonts (dividing forms) may be obseved, though larger schizonts of greater nuclear count are not reliably presented. Cells are often phagocytosed by host haemocytes. *Perkinsus marinus* cells stain basophilic.
- In susceptible host species, within the known range of *P. marinus*, a positive result is presumptive evidence of *P. marinus* 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 *P. chesapeaki* or undescribed *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. marinus.* The technique is simple, inexpensive and very sensitive, but not species specific. Trophozoites of *P. marinus* in oyster 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 become easily visible at low power because of their bluish–black colouration and 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 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. 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 (whole body burden assay) is the gold standard. The fluid thioglycollate culture tissue assay is the recommended surveillance method. The method has been validated against the whole body burden assay (Bushek *et al.,* 1994) and shown to be less sensitive.

Interpretation of results:

- Cultured parasites enlarge from 2–10 to 20–70 µm during incubation. *Perkinsus* spp. cells are spherical and the walls stain blue or bluish-black with Lugol's iodine solution (Bushek *et al.,* 1994; Ray 1966).
- In susceptible host species, within the known range of *P. marinus*, a positive result is presumptive evidence of *P. marinus* 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 *P. chesapeaki* or undescribed *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 marinus cells are easily cultured in a variety of media (e.g. La Peyre *et al.,* 1993). Culture medium is usually inoculated with heart, haemolymph, or gill tissue. Comparisons of commercially available media have been made (Dungan & Hamilton, 1995) and growth was supported in all media, but was at a maximum in 1/1 DME (Dulbecco's Modified Eagle's)/Ham's F-12 medium.

4.3.1.2.2. Antibody-based antigen detection methods

Monoclonal and polyclonal antibodies have been developed for *P. marinus*, but they are not specific to *P. marinus* and the polyclonal antibody has been shown to cross react with some dinoflagellate species (Bushek *et al.,* 2002a).

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. marinus* specific assay. Much more is known about inter- and intra-specific sequence variation of the internal transcribed spacer (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. marinus* strains. For *in-situ* hybridisation (ISH), probes have been developed that target the small subunit (SSU) gene 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 has been shown to be more specific in a limited validation against the RFTM assay. This assay needs to be tested more thoroughly for inclusivity, 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 a mollusc infected with any *Perkinsus* sp. 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 then 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 (Elston *et al*., 2004).

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

4.3.1.2.3. Perkinsus marinus-*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. marinus*. 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 (Audemard *et al.,* 2004). The most recent version of the recommended specific *P. marinus* ITS assay is presented here. It is recommended that surveillance using the *Perkinsus* genus assay be conducted first, and then the specific assay. In addition, a real-time PCR assay has been developed for *P. marinus* in host tissue (Gauthier *et al.,* 2006). It has been tested only with *P. marinus, P. olseni* and *P. chesapeaki,* and has been shown to be more sensitive in a limited validation against the RFTM. This assay needs to be tested more thoroughly for specificity with all known *Perkinsus* species, but may be useful for laboratories that possess the necessary equipment.

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. marinus* rRNA gene complex have been developed (Audemard *et al.,* 2004): forward primer PmarITS-70F (5'-CTT-TTG-YTW-GAG-WGT-TGC-GAG-ATG-3') and reverse primer PmarITS600R (5'-CGA-GTT-TGC-GAG-TAC-CTC-KAG-AG-3'). The amplified product size is 509 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, 0.05 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, 57°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. marinus* 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. olseni*, *P. chesapeaki, P. andrewsi* and *P. mediterraneus*, as well as a number of dinoflagellate species (Audemard *et al.,* 2004). Sensitivity is high with the ability to detect one *P. marinus* 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 has not been validated against the RFTM.

Interpretation of results:

- A positive result is a PCR amplification product of the appropriate size (509 bp), with all negative controls negative and all positive controls positive.

Availability of commercial tests: not commercially available.

Also, a PCR-restriction fragment length polymorphism (RFLP) assay has been developed that may be useful for specific diagnoses of *P. marinus* (Abollo *et al.,* 2006), although it has not been tested for specificity against all known *Perkinsus* species.

4.3.1.2.3.4. Perkinsus marinus-*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. marinus* has been developed (Moss *et al.,* 2006) (PmarLSU-181DIG 5'-GAC-AAA-CGG-CGA-ACG-ACT-C-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 *C. virginica* infected with *P. marinus*. Negative controls are either no-probe assays or assays with uninfected oysters.

Levels of validation:

- *Specificity and sensitivity:* the *P. marinus* DNA probe has been tested for specificity against a variety of *Perkinsus* species (Moss *et al.,* 2006). 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 marinus 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 marinus* 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 infections visualised by smear, RFTM or histology; PCR = polymerase chain reaction.

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

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

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

In known susceptible species, within the known geographical range of *P. marinus*, a suspect case of infection with *P. marinus* 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. marinus*, 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. marinus* 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.

8. References

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NB: Currently (2021) there is no OIE Reference Laboratory for Infection with *Perkinsus marinus* (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 2012.