Annex 28. Item 10.1.2. Chapter 2.4.6. ‘Infection with *Perkinsus olseni’*

Chapter 2.4.6.

infection with *Perkinsus olseni*

1. Scope

Infection with *Perkinsus olseni* is considered to be infection with *Perkinsus. olseni* (Family: *Perkinsidae)*

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

The aetiological agent is the protozoan parasite *Perkinsus olseni*.

Current evidence using genomic sequencing data have placed *Perkinsus olseni* in the class Perkinsea of the phylum Perkinsozoa, and Kingdom Alveolata. *Perkinsus olseni* was formerly known as *Perkinsus atlanticus* in Europe (Azevedo 1989).

As trophozoites grow, cleavage furrows begin to form on the cell surface, signaling the early stages of their subdivision, or the onset of schizogony (Gajamange *et al.,* 2020). As cleavage advances, the number of daughter cells increases, progressing to the morula stage, where approximately 100 irregularly shaped merozoites are observed. Once schizonts are fully developed, they release hundreds of merozoites upon the rupture or abrasion of the schizont cellular membrane.

A study investigating genome-wide comparison of five *P. olseni* isolates from Australia (00978-12), New Zealand (PRA 205 and 207), Japan (PRA-179), and Spain (PRA-31) observed differences between different geographical locations. Indeed, *P. olseni* from Oceania (Australia and New Zealand) displayed a high heterozygosity of 5.47–7.71% compared with Eurasia (Spain and Japan) with 0.1 to 0.2% of heterozygosity (Bogema *et al.,* 2021). The gene numbers differed substantially, with the Oceanian isolates sharing a high proportion of unique orthogroups in comparison to the Eurasian isolates (Bogema *et al.,* 2021). The Oceanian isolates had a slightly higher proportion of repetitive content such as tandem gene duplication. The characterised gene contents appear to be highly conserved across *Perkinsus* species such as in *P. olseni*, *P. marinus*, and *P. chesapeaki* (Bogema *et al.,* 2021).

The ribosomal RNA transcription unit is highly duplicated with variations between the isolates from different parts of the world, which does not make it a suitable region for designing real-time PCR tests to assess the intensity of infection (Bogema *et al.,* 2021).

More studies are needed to confirm the ploidy of *P. olseni*. Indeed, some authors using genome-wide data suggested that *Perkinsus* is polyploid with a ploidy variation between individual cells and populations (Bogema *et al.,* 2021) whereas other using microsatellite loci or restriction fragment length polymorphism proposed a diploidy (Pardo *et al.,* 2011; Reece *et al.,* 1997; 2001; Robledo *et al.,* 1999; Thompson *et al.,* 2011).

2.1.2. Survival and stability in processed or stored samples

*Perkinsus olseni* can be propagated *in vitro* in various media formulations such as Dulbecco’s Modified Eagle medium and Ham’s F-12 nutrient mixture (Burreson *et al.,* 2005; Dungan & Reece, 2006) or JL-ODR-2A (La Peyre *et al.,* 2006). Both media are supplemented with various salts, FBS, lipid mixtures, and yeast ultrafiltrates. Isolates can be cryopreserved and stored indefinitely (Dungan et al., 2007).

2.1.3. Survival and stability outside the host

Long-term survival of *P. olseni* outside its bivalve host is not known. but it is at least 4 months for prezoosporangia (Casas *et al.,* 2002b).

2.2. Host factors

NB: Sections 2.2.1 and 2.2.2 are not for Member comment. Version for comment and adoption are presented in Annex 26

~~2.2.1. Susceptible host species~~

~~Species that fulfil the criteria for listing as susceptible to infection with~~ *~~Perkinsus olseni~~* ~~according to Chapter 1.5. of the~~ *~~Aquatic Animal Health Code~~* ~~(~~*~~Aquatic Code~~*~~) are:~~

| ~~Family~~ | ~~Scientific name~~ | ~~Common name~~ |
| --- | --- | --- |
| ~~Arcidae~~ | *~~Anadara kagoshimensis~~* | ~~half-crenated ark~~ |
|  | *~~Anadara trapezia~~* | ~~ark cockle~~ |
| ~~Cardiidae~~ | *~~Tridacna crocea~~* | ~~crocus giant clam~~ |
| ~~Haliotidae~~ | *~~Haliotis laevigata~~* | ~~greenlip abalone~~ |
|  | *~~Haliotis rubra~~* | ~~blacklip abalone~~ |
| ~~Margaritidae~~ | *~~Pinctada fucata~~* | ~~Japanese pearl oyster~~ |
| ~~Mytilidae~~ | *~~Mytilus galloprovincialis~~* | ~~Mediterranean mussel~~ |
|  | *~~Perna canaliculus~~* | ~~New Zealand mussel~~ |
| ~~Veneridae~~ | *~~Austrovenus stutchburyi~~* | ~~Stutchbury’s venus clam~~ |
|  | *~~Leukoma jedoensis~~* | ~~Jedo venus clam~~ |
|  | *~~Paratapes undulatus [syn. Paphia ondulata]~~* | ~~undulate venus clam~~ |
|  | *~~Protapes gallus~~* | ~~rooster venus clam~~ |
|  | *~~Proteopitar patagonicus~~* | ~~no common name~~ |
|  | *~~Ruditapes decussatus~~* | ~~grooved carpet shell~~ |
|  | *~~Ruditapes philippinarum~~* | ~~Manila clam~~ |

~~2.2.2. Species with incomplete evidence for susceptibility~~

~~Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with~~ *~~P. olseni~~* ~~according to Chapter 1.5. of the~~ *~~Aquatic Code~~* ~~are:~~

| ~~Family~~ | ~~Scientific name~~ | ~~Common name~~ |
| --- | --- | --- |
| ~~Cardiidae~~ | *~~Cerastoderma edule~~* | ~~common edible cockle~~ |
| ~~Mytilidae~~ | *~~Mytilus chilensis~~* | ~~Chilean mussel~~ |
| ~~Ostreidae~~ | *~~Crassostrea gasar~~* | ~~gasar cupped oyster~~ |
|  | *~~Ostrea angasi~~* | ~~Australian mud oyster~~ |
| ~~Pectinidae~~ | *~~Pecten novaezelandiae~~* | ~~New Zealand scallop~~ |
| ~~Psammobiidae~~ | *~~Hiatula acuta~~* | ~~no common name~~ |
| ~~Veneridae~~ | *~~Venerupis corrugata~~* | ~~corrugated venus clam~~ |

~~In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated:~~

| ~~Family~~ | ~~Scientific name~~ | ~~Common name~~ |
| --- | --- | --- |
| ~~Arcidae~~ | *~~Tegillarca granosa~~* | ~~blood cockle~~ |
|  | *~~Scapharca subcrenata~~* |  |
| ~~Cardiidae~~ | *~~Cerastoderma glaucum~~* | ~~olive green cockle~~ |
| ~~Chamidae~~ | *~~Chama pacifica~~* | ~~reflexed jewel box~~ |
| ~~Haliotidae~~ | *~~Haliotis iris~~* | ~~New Zealand black-footed abalone~~ |
| *~~Haliotis diversicolor~~* | ~~small abalone~~ |
| ~~Isognomonidae~~ | *~~Isognomon alatus~~* | ~~flat tree oyster~~ |
|  | *~~Isognomon~~* ~~sp.~~ | ~~N/A~~ |
| ~~Margaritidae~~ | *~~Pinctada imbricata~~* | ~~Atlantic pearl oyster~~ |
| ~~Ostreidae~~ | *~~Crassostrea rhizophorae~~* | ~~mangrove cupped oyster~~ |
|  | *~~Dendostrea frons~~* | ~~Frons oyster~~ |
|  | *~~Magallana~~* ~~[syn.~~ *~~Crassostrea~~*~~]~~ *~~gigas~~* | ~~Pacific oyster~~ |
|  | *~~Magallana~~* ~~[syn.~~ *~~Crassostrea~~*~~]~~ *~~hongkongensis~~* | ~~no common name~~ |
|  | *~~Saccostrea~~* ~~sp.~~ | ~~N/A~~ |
| ~~Pectinidae~~ | *~~Mimachlamys crassicostata~~* | ~~noble scallop~~ |
|  | *~~Chlamys nobilis~~* | ~~noble scallop~~ |
| ~~Pharidae~~ | *~~Sinonovacula constricta~~* | ~~constricted tagelus clam~~ |
| ~~Solenidae~~ |  |  |
| ~~Veneridae~~ | *~~Meretrix lyrata~~* | ~~lyrate hard clam~~ |
|  | *~~Polititapes aureus~~* | ~~golden carpet shell~~ |
|  | *~~Venus verrucosa~~* | ~~warty venus clam~~ |

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

*Perkinsus olseni* is known to infect and cause clinical signs in many bivalve species. All stages after settlement are susceptible. *P. olseni* infection intensity increases with host age (Villalba *et al.,* 2005).

2.2.4. Distribution of the pathogen in the host

*Perkinsus olseni* trophozoites and schizonts can be found in various tissues and organs, including connective tissues, siphons, mantle, gills, digestive system, foot, adductor muscle, and haemolymph (Carella *et al.,* 2023; Park & Choi, 2001; Leethochavalit *et al.,* 2004). In some clams, the infection was heaviest in the digestive gland and gills (Leethochavalit *et al.,* 2004; Park & Choi, 2001). However, infection in the gills of bivalves is representative of the infection throughout the entire body and is therefore considered the organ of choice for diagnosing *P. olseni* infection (Choi *et al.,* 2002; Cui *et al.,* 2018; Dang *et al.,* 2013; Park *et al.,* 1999). *Perkinsus olseni* has also been detected in faeces of infected animals (Park *et al.,* 2010).

2.2.5. Aquatic animal reservoirs of infection

None known.

2.2.6. Vectors

None known.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Infections in clam hosts can be lethal depending on environmental conditions, and death may occur 1 or 2 years after infection. Studies have suggested that the impact on the host depends on the intensity of infection but also on host species and locations; it can start being deleterious and lethal at densities of 106 parasite cells/g wet tissue (Dang *et al.,* 2013; Waki *et al.,* 2018).

Prevalence is highly variable depending on host and environmental conditions, up to 100% in susceptible host species as determined by histology or polymerase chain reaction (PCR). Prevalence and intensity of infection may be higher in individuals with more than 1 year of exposure to the pathogen because they have been exposed to *P. olseni* for longer, and larger animal have a higher filtration rate (Choi *et al.,* 2002; Villalba *et al.,* 2005). Prevalence can vary according to the season and is often higher in spring (Cui *et al.,* 2018; Villalba *et al.,* 2005).

2.3.2. Clinical signs, including behavioural changes

*Perkinsus olseni* infection can lead to reduced growth, reproduction issues, and mass mortalities in shellfish populations (Cho & Park, 2010). Clinical signs include mantle retraction, byssal tissue sloughing, gaping or dead molluscs but these clinical signs are not specific to infection with *P. olseni.* Individual bivalves with late-stage infections may exhibit slow responses to stimuli (Sheppard & Phillips, 2008).

2.3.3 Gross pathology

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

At high infection intensities the infected molluscs can present several milky-white cysts, abscesses or brown nodules (Azevedo, 1989; Gudkovs *et al.,* 2016; Ruano *et al.,* 2015). The cysts or nodules contain individual and grouped encapsulated trophozoites at different stages of maturation, and result from a natural defensive reaction, the infiltration of hemocytes (Abdel-Baki *et al.,* 2014).

2.3.4. Modes of transmission and life cycle

The life cycle is horizontal, direct from host to host (Villalba *et al.,* 2004). Faecal discharge and decomposition of infected tissues after the host death have been suggested as two transmission pathways for *P. olseni* (Cui *et al.,* 2018; Park *et al.,* 2010).

The life cycle consists of three main life stages: trophozoite, prezoosporangium, and zoospore (Villalba *et al.,* 2004). The trophozoite is a stage occurring in the tissues of the live host where vegetative proliferation occurs. The trophozoite undergoes successive bipartitioning to yield up to 32 daughter cells that stay together in a rosette-like arrangement inside a wall. After rupture of the wall, immature trophozoites enlarge and form a vacuole, becoming mature trophozoites. At the host death, trophozoites evolve into a prezoosporangia, which evolve into zoosporangia when they are released in the environment. Zoosporangia produces zoospores, which are the infective stage.

2.3.5. Environmental factors

Environmental factors such as temperature, salinity, oxygen levels, pH, and nutrient availability influence *Perkinsus* infection dynamics and hypnospore formation (2013; Villalba *et al.,* 2004).

Temperature and salinity appear to be the most important environmental factors controlling the transmission of *P. olseni* infection with zoosporulation occurring between 19 and 28°C and increasing with higher temperature and salinity (Kyoung & Ki, 2001; Umeda *et al.,* 2020).

Temperature and salinity also appear to be the two major environmental factors influencing the prevalence and seasonality of *P. olseni* infection, with several studies reporting higher prevalence and intensity of infection in spring (Park & Choi 2001; Soudant *et al.,* 2013; Villalba *et al.,* 2005; Waki & Yoshinaga, 2013; 2015).

2.3.6. Geographical distribution

Infections are widespread throughout the tropical Pacific Ocean, Oceania, Asia, Europe and South America (Cremonte *et al.,* 2005; Goggin & Lester, 1995; Villalba *et al.,* 2004). *Perkinsus olseni* is not known from North America.

See WAHIS (<https://wahis.woah.org/#/home>) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

None.

2.4.2. Chemotherapy including blocking agents

Cyclohexamide, pyrimethamine, deferoxamine (DFO) and 2, 2-bipyridyl inhibit *P. olseni* development/replication *in vitro*, and DFO inhibits *P. olseni* development/infection *in vivo*. (Elandalloussi *et al.,* 2005).

2.4.3. Immunostimulation

None.

2.4.4. Breeding resistant strains

None.

2.4.5. Inactivation methods

Isolated *P. olseni* cells were killed when immersed in freshwater within 10 minutes at room temperature. Another study showed that free zoospores, free prezoosporangia and prezoosporangia in gill tissues were killed after 1 hour incubation with 50, 200, and 3000 ppm of chlorine, respectively (Casas *et al.,* 2002b). *Perkinsus olseni* cells in host tissue were much more resistant to these treatments. UV-C irradiation has been shown to be effective in inactivating *Perkinsus* parasites, with a minimum dose of 94 mJ/cm2 required to inhibit proliferation and 450 mJ/cm2 to completely kill all parasites (Fernandez-Boo *et al.,* 2021).

2.4.6. Disinfection of eggs and larvae

*Perkinsus olseni* is not known to infect eggs or larvae of its hosts, but parasite cells may occur intercellularly.

2.4.7. General husbandry

Management strategies to mitigate perkinsosis impacts include modifying culture procedures such as reducing stocking density, selective breeding for resistant strains, and using triploid or allochthonous oyster species (Villalba *et al.,* 2004).

3. Specimen selection, sample collection, transportation and handling

This section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples that are most likely to be infected.

3.1. Selection of populations and individual specimens

Gaping, clams that are at the surface instead of being buried, or freshly dead animals should be targeted to increase the chances of finding infected animals. Abalone with foot blisters should be sampled. For histology, only live animals should be taken.

3.2. Selection of organs or tissues

Connective tissue of all organs, and haemocytes.

For RFTM, the whole animal should be ideally sampled if its size allows. If not, pieces of gill followed by mantle or foot for abalone are typically used.

For culture purposes, tissue slices that can include gill, mantle, or foot (for abalone) can be 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 recommended.

3.3. Samples or tissues not suitable for pathogen detection

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

3.4. Non-lethal sampling

For *P. marinus*, Gauthier & Vasta (1995) proposed haemolymph incubation in Ray’s Fluid Thioglycollate Medium (RFTM) as an alternative to the traditional tissue RFTM, enabling non-lethal sampling of individuals. This method was further refined by Nickens *et al.* (2002). Rodriguez & Navas (1995) reviewed and compared various RFTM assays for *Perkinsus olseni*, highlighting that whole host body incubation in RFTM is the most sensitive method.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.4.0 General information (diseases of molluscs).

3.5.1. Samples for pathogen isolation

Theresults of bioassay depend strongly on the quality of samples (time since collection, time in storage, preservative used). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples, use alternative storage methods only after consultation with the receiving laboratory.

3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 80% (v/v) analytical-grade ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5 of Chapter 2.4.0 *General information* (diseases of molluscs).

3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.5.3 of Chapter 2.4.0 *General information* (diseases of molluscs).

3.5.4. Samples for other tests

None.

3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose is only recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger specimens should be processed and tested individually. Small life stages such as spat can be pooled to obtain the minimum amount of material for molecular detection. Pooling of very small spat (5–10 depending on size) is acceptable for PCR analyses.

4. Diagnostic methods

The methods currently available for pathogen detection that can be used in i) surveillance of apparently healthy animals, ii) presumptive diagnosis in clinically affected animals and iii) confirmatory diagnostic purposes are listed in Table 4.1. by animal life stage.

**Ratings for purposes of use.** For each recommended assay a qualitative rating for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

+++ = Methods are most suitable with desirable performance and operational characteristics.

++ = Methods are suitable with acceptable performance and operational characteristics under most circumstances.

+ = Methods are suitable, but performance or operational characteristics may limit application under some circumstances.

Shaded boxes = Not appropriate for this purpose.

**Validation stage**. The validation stage corresponds to the assay development and validation pathway in chapter 1.1.2. The validation stage is specific to each purpose of use. Where available, information on the diagnostic performance of recommended assays is provided in Section 6.3.

WOAH Reference Laboratories welcome feedback on diagnostic performance of recommended assays, in particular PCR methods. Of particular interest are any factors affecting expected assay sensitivity (e.g. tissue components inhibiting amplification) or expected specificity (e.g. failure to detect particular genotypes, detection of homologous sequences within the host genome). These issues should be communicated to the WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals

| Method | 1. Surveillance of apparently  healthy animals | | | | 1. Presumptive diagnosis of  clinically affected animals | | | | 1. Confirmatory diagnosis1 of a suspect result from surveillance or presumptive diagnosis | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Early life stages2 | Juveniles2 | Adults | LV | Early life stages2 | Juveniles2 | Adults | LV | Early life stages2 | Juveniles2 | Adults | LV |
| Wet mounts |  |  |  |  |  |  |  |  |  |  |  |  |
| Histopathology |  | ++ | ++ | 2 |  | +++ | +++ | NA |  |  |  |  |
| Cell culture |  |  |  |  |  |  |  |  |  |  |  |  |
| Transmission electron microscopy |  |  |  |  |  |  |  |  |  |  |  |  |
| Real-time PCR | +++ | +++ | +++ | 3 | +++ | +++ | +++ | NA | +++ | +++ | +++ | 3 |
| Conventional PCR | +++ | +++ | +++ | 3 | +++ | +++ | +++ | NA |  |  |  |  |
| Conventional PCR followed by amplicon sequencing |  |  |  |  |  |  |  |  | +++ | +++ | +++ | 3 |
| *In-situ* hybridisation |  |  |  |  |  |  |  |  |  | +++ | +++ | 3 |
| Bioassay |  |  |  |  |  |  |  |  |  |  |  |  |
| LAMP |  |  |  |  |  |  |  |  |  |  |  |  |
| Ab-ELISA |  |  |  |  |  |  |  |  |  |  |  |  |
| Ag-ELISA |  |  |  |  |  |  |  |  |  |  |  |  |
| RFTM |  | +++ | +++ | 3 |  | +++ | +++ | 3 |  |  |  |  |

LV = level of validation, refers to the stage of validation in the WOAH Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification;   
Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; IFAT = indirect fluorescent antibody test. [give definitions of abbreviations as appropriate; nPCR = nested PCR, etc. NB “RT-PCR” is reserved for reverse-transcription polymerase chain reaction methods. “real-time PCR” should always be stated in full and refers to probe-based and SYBR green assays]   
1For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). 2Susceptibility of early and juvenile life stages is described in Section 2.2.3.   
3Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Smears

Not recommended as a diagnostic method.

4.2. Histopathology

Samples to be taken consist of live or moribund bivalves.

*S*ections of tissues that include gills, digestive gland and mantle should be fixed for a minimum of 24 hours in a recommended fixative followed by standard processing for histology as described in Section 5.3 of Chapter 2.4.0 *General information* (diseases of molluscs). Observations are made at increasing magnifications up to ×400.

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).

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, may indicate infection with *Perkinsus olseni*. 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.

4.3. Electron microscopy

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

4.4. Nucleic acid amplification

Samples to be taken consist of live or freshly dead molluscs. 2–3 mm2 tissue pieces are excised aseptically from gill and mantle and placed into 1.5 ml microcentrifuge tubes containing 80% ethanol. Dissecting utensils should be flamed between samples to prevent cross-contamination.

PCR assays should always be run with the controls specified in Section B.5.5 *Molecular methods* Chapter 2.4.0 *General information* (diseases of molluscs). Each sample should be tested in duplicate.

*Extraction of nucleic acids*

DNA is extracted by proteinase K digestion overnight at 56°C and the spin-column methodology using commercially available kits. Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

4.4.1. Real-time PCR

A real-time *Perkinsus* genus PCR assay targeting the ITS region 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.

Several real-time PCR assays all targeting the ITS region have been developed to detect and quantify *P. olseni* (Cui *et al.,* 2018; Gajamange *et al.,* 2011; Itoïz *et al.,* 2021; Ríos *et al.,* 2020; Umeda & Yoshinaga, 2012). One needs to be careful when attempting to quantify *P. olseni* as genome sequencing revealed that this region is highly duplicated, which does not make it the ideal region for quantifying parasite infection. Of all those assays, only the assay developed by Ríos *et al.* (2020) presented some level of validation such as sensitivity and reproducibility. More validation is required before a real-time PCR assay can be recommended (see chapter 1.1.2 *Principles and methods of validation of diagnostic assays for infectious diseases*). In addition, Ríos *et al.* (2020) detected non-specific hybridisation when using the Umeda & Yoshinaga (2012) and Gajamange *et al.* (2011) assays.

***Primers and probes* (*sequences*)**

Method 1: TaqMan PCR Gauthier *et al.* (2006); GenBank Accession No.: AF149876.1

Method 2: TaqMan PCR Itoiz *et al.* (2021); GenBank Accession No.: MW187111

Method 3: SYBR Green PCR Rios *et al.* (2020); GenBank Accession No.: ???

Method 4: Cui *et al*. (2018); GenBank Accession No.: ???; amplicon size [217 bp]

Method 6: Umeda &Yoshinaga, (2012); GenBank Accession No.: ???

Method 5: Gajamange *et al.* (2011); GenBank Accession No.: ???

|  |  |  |  |
| --- | --- | --- | --- |
| Pathogen/ target gene | Primer/probe (5’–3’)  Concentration  Cycling parameters(a) | | |
| *Perkinsus* sp./ITS  (detects at least *P. marinus, P. olseni* and *P. chesapeaki*).  Method 1 | PERK-F: CGT-GAA-CCA-GTA-GAA-ATC-TCA-A PERK-R: ACA-TAT-CAG-TGT-CGC-TCT-TCT-TCC Probe: GCA-TAC-TGC-ACA-AAG-GG  0.9 µM 0.9 µM 0.25 µM  40 cycles of: 95°C/15 sec and 60°C/60 sec | | |
| *P. olseni*  Method 2 | PolsITS2-F: CAC-CAC-AAC-ACA-GTC-GGA-C PolsITS2-R: CGT-ATT-GTA-GCC-CCT-CCG-A PolsITS2-probe: GAC-ACT-CAC-AGG-CGC-GGT-CC  0.2 µM 0.2 µM 0.5 µM  45 cycles of: 95°C/10 sec and 55°C/20 sec | | |
| *P. olseni*  Method 3 | Perk-ITS-qF1: CTG-ACC-GCC-TTA-ACG-GGC Perk-ITS-qR2: CTA-TCT-CCG-AAG-AGT-TAG-TCC-  10 µM 10 µM  40 cycles of: 95°C/30 sec and 60°C/60 sec | | |
| *P. olseni / ITS*  Method 4 | PO-F: GAG-TGT-CTC-TGG-TTG-CTC-GCA PO-R: ACA-TCA-GGC-CTT-CTA-ATG-ATG  10 µM 10 µM  40 cycles of: 95°C/15 sec and 60°C/60 sec | | |
| *P. olseni*  Method 6 | OF: CTT-AAC-GGG-CCG-TGT-TA OR: CAT-AAC-GAA-CTA-TCT-CCG-AAG  0.5 µM 0.5 µM  40 cycles of: 98°C/2 sec and 60°C/5 sec | | |
| *P. olseni*  Method 5 | PK-ITS-F: CAG-AAT-TCC-GTG-AAC-CAG-TAG-A PK-ITS-R: TGT-CGC-TCT-TCT-TCC-CGA-TA Probe: TCA-ACG-CAT-ACT-GCA-CAA-AGG-GGA | 10 pM 10 pM 10 pM | 40 cycles of: 95°C/10 sec and 56°C/30 sec |

(a)A denaturation step prior to cycling has not been included.

4.4.2. Conventional PCR

Method 1: Moss *et al.,* 2006; GenBank Accession No.: ???; amplicon size: [bp] ???

Method 2: Casas *et al.,* 2002a; GenBank Accession No.: ???; amplicon size: [bp] ???

|  |  |  |  |
| --- | --- | --- | --- |
| Pathogen/ target gene | Primer/probe (5’–3’) | Concentration | Cycling parameters(a) |
| *P. olseni*  M*ethod 1* | PolsITS-140F: GAC-CGC-CTT-AAC-GGG-CCG-TGT-T PolsITS-600R: GGR-CTT-GCG-AGC-ATC-CAA-AG 450 bp  0.1 µM 0.1 µM  40 cycles of: 94°C/60 sec and 62°C/60 sec and 65°C/180 sec | | |
| *Perkinsus* spp. (except *P. qugwadi*)  Method 2 | PerkITS-85: CCG-CTT-TGT-TTG-GAT-CCC PerkITS-750: ACA-TCA-GGC-CTT-CTA-ATG-ATG 675bp product | 0.1 µM 0.1 µM | 40 cycles of: 95°C/1 min and 55°C/1 min and 72°C/1 min |

(a)A denaturation step prior to cycling has not been included.

4.4.3. Other nucleic acid amplification methods

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.

A LAMP assay was developed by (Feng *et al.,* 2013) targeting ITS-2 and appeared to be rapid, sensitive (detection limit of 10 copies of plasmid DNA), and specific for *Perkinsus* spp. detection.

4.5. Amplicon sequencing

The size of the PCR amplicon should be verified, for example by agarose gel electrophoresis. Both DNA strands of the PCR product must be sequenced and analysed in comparison with reference sequences.

4.6. *In-situ* hybridisation

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

For *in-situ* hybridisation (ISH), probes have been developed that target the small subunit (SSU) of the rRNA gene complex (Elston *et al.,* 2004).

**Perkinsus genus-specific *in-situ* hybridisation**

Samples to be taken: follow the procedure for ‘fixed sections’ 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).

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 MgCl2) 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 (dH2O). The sections are counterstained with Bismarck Brown Y, rinsed in dH2O, 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.

**Perkinsus olseni-specific *in-situ* hybridisation**

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 arecompulsory. 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.

*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’).

Muznebin *et al.,* (2023) also used ISH using the DIG PCR probe synthesis kit (Roche). A DIG-labelled probe was generated using the PCR primers Pols140F (5’-GAC-CGC-CTT-AAC-GGG-CCG-TGT-T-3’) and PolsITS-600R (5’-GGR-CTT-GCG-AGC-ATC-CAA-AG-3’) (Moss *et al.,* 2006). The DIG-labelled probe was used at a concentration of 5 ng/µl.

4.7. Immunohistochemistry

Not available.

4.8. Bioassay

Not available.

4.9. Antibody~~-~~ or antigen-based detection methods (ELISA, etc.)

Not currently available for diagnostic purposes but monoclonal antibodies have been developed (Hanrio et al., 2021; 2022; Park et al., 2010;).

4.10. Other methods

4.10.1. 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 supplemented 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 consist of live or freshly dead molluscs.

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 (dH2O), 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 dH2O; 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; dH2O, 100 ml. Lugol’s iodine working solution: dH2O, 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.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time PCR and RFTM tissue or whole-body burden assays are recommended for targeted surveillance to declare freedom from infection with *P. olseni*.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1.) or in the presence of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free. There are currently no WOAH Reference Laboratory for *Perkinsus olseni*.

6.1. Apparently healthy animals or animals of unknown health status[[1]](#footnote-2)

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Hydrographical proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with *P. olseni* shall be suspected if at least one of the following criteria is met:

i) Histopathological changes consistent with the presence of the pathogen or the disease

ii) Positive result by conventional PCR

iii) Positive result by real-time PCR

iv) Positive result by RFTM

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *P. olseni* is considered to be confirmed if the following criterion is met:

1. Positive result by real-time PCR and positive result by conventional PCR followed by amplicon sequencing
2. Positive result by *In-situ* hybridisation and positive result by conventional PCR followed by amplicon sequencing
3. Positive result by *In-situ* hybridisation and positive result by species specific real-time PCR.

6.2 Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *P. olseni* shall be suspected if at least one of the following criteria is met:

1. Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality
2. Histopathological changes consistent with the presence of the pathogen or the disease
3. Positive result by conventional PCR
4. Positive result by real-time PCR
5. Positive result by RFTM

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *P. olseni* is considered to be confirmed if at least one of the following criteria is met:

i) Positive result by real-time PCR and conventional PCR followed by sequence analysis

ii) Positive result ISH and conventional PCR followed by sequence analysis

ii) Positive result of real-time PCR and ISH

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *P. olseni* are provided in Tables 6.3.1. and 6.3.2 (no data are currently available for either). Data are only presented where tests are validated to at least level 2 of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

6.3.1. For presumptive diagnosis of clinically affected animals (no data are currently available)

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|  |  |  |  |  |  |  |  |  |

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of animals used in the validation study,  
PCR: = polymerase chain reaction.

6.3.2. For surveillance of apparently healthy animals (no data are currently available)

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|  |  |  |  |  |  |  |  |  |

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of animals used in the validation study,  
PCR: = polymerase chain reaction.

7. References

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\*  
\* \*

**NB:** Currently (2025) there is no WOAH Reference Laboratory for infection with *Perkinsus olseni*  
(please consult the WOAH web site:   
<https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

**NB:** First adopted in 1995 as Perkinsosis. Most recent updates adopted in 2015.

1. For example transboundary commodities. [↑](#footnote-ref-2)