CHAPTER 2.4.4.

INFECTION WITH *MARTEILIA REFRINGENS*

1. Scope

Marteilia refringens is a protozoan parasite of the phylum Cercozoa and order Paramyxida (Cavalier-Smith & Chao, 2003; Feist *et al.*, 2009) infecting the digestive system of several bivalve species and inducing physiological disorders and eventually death of the animal (Alderman, 1979; Grizel *et al*., 1974). For the purpose of this chapter, infection with *Marteilia refringens* covers infection with *M. refringens* as defined by Lopez-Flores *et al.* (2004) including types M and O as defined by Le Roux *et al*. (2001). This definition excludes infections with *M. sydneyi* (Perkins & Wolf, 1976), *M. lenghei* (Comps, 1976) and *M. christenseni* (Comps, 1983). *Marteilia* spp. that are not identified to the species level (Berthe *et al*., 2004; Moyer *et al*., 1993; Norton *et al*., 1993) should be referred to the appropriate WOAH Reference Laboratory.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Marteilia refringens (Grizel *et al*., 1974), two types, types O and M, were defined by Le Roux *et al.* (2001).

2.1.2. Survival outside the host

Depending on the environmental conditions, *M. refringens* can survive from several days up to 2–3 weeks outside the host (Grizel, 1985).

2.1.3. Stability of the agent (effective inactivation methods)

No data available.

2.1.4. Life cycle

The life cycle of *M. refringens* is supposed to be indirect and may include *Paracartia grani* (Audemard *et al.,* 2001; 2002), at least in pond systems. In other species, including other *Acartia* spp., the cyclopoida *Oithona* sp. and an indeterminate harpaticoida species, the parasite has been detected by polymerase chain reaction (PCR) in the natural estuary of the Ebro Delta (Spain), but their role in the life cycle has not been demonstrated (Carrasco *et al.,* 2007).

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *Marteilia refringens* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: blue mussel (*Mytilus edulis*), dwarf oyster (*Ostrea stentina*), European flat oyster *(Ostrea edulis)*, European razor clam (*Solen marginatus*), golden mussel (*Xenostrobus securis*), Mediterranean mussel (*Mytilus galloprovincialis*) and striped venus clam (*Chamelea gallina*).

Additionally, a copepod species (*Paracartia grani*) has been found to meet the criteria for listing as susceptible to infection with *Marteilia refringens* and is considered an intermediate host

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 *M. refringens* according to Chapter 1.5. of the *Aquatic Code* are: Chilean flat oyster

(*Ostrea chilensis*), a copepod (*Paracartia latisetosa*) and Japanese flat oyster (*Ostrea denselamellosa*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: Cortez oyster (*Crassostrea corteziensis*), grooved carpet shell (*Ruditapes decussatus*), Pacific cupped oyster (*Magallana* [syn. *Crassostrea] gigas*) and zooplankton (*Acartia discaudata, Centropages typicus*, *Euterpina acutifrons*, unidentified *Oithona sp*., *Penilia avirostris*).

2.2.3. Species or subpopulation predilection (probability of detection)

Marteilia refringens usually causes clinical infection in *O. edulis* (Grizel *et al*., 1974) and *Ostrea* spp. (Berthe *et al*., 2004; Grizel, 1985). In flat oysters and mussels, prevalence and infection intensity are generally higher in individuals of 2 years of age or more (Audemard *et al.,* 2001; Villalba *et al*., 1993b).

2.2.4. Target organs and infected tissue

Marteilia refringens infects the digestive tract. Young plasmodia are mainly found in the epithelium of labial palps and the stomach (Grizel *et al*., 1974). Sporulation takes place in the digestive gland tubules and ducts. Propagules are released into the lumen of the digestive tract and shed into the environment in faeces (Audemard *et al.,* 2002; Berthe *et al*., 2004).

2.2.5. Persistent infection with lifelong carriers

Infection with *M. refringens* is a lethal disease of oysters (Alderman, 1979; Audemard *et al.,* 2002; Grizel *et al*., 1974). Death occurs during the second year after initial infection (Alderman, 1979; Grizel, 1985), so infection may persist for more than 1 year and may be lifelong. Mussels are usually not adversely affected by *M. refringens* (Berthe *et al*., 2004), but whether sporulation of *M. refringens* occurs and whether mussels can be carriers of *M. refringens* are not known (Berthe *et al*., 2004; Le Roux *et al*., 2001).

2.2.6. Vectors

Several zooplankton species, including copepod species (*Acartia discaudata*, *A. clausi*, *A. italica*, *Othoina* sp., *Euterpina acutifrons*) and zoeal larval stages of Brachyuran decapods, as well as non-planktonic species, such as *Lineus gisserensis* (Nematoda) and *Cereus pendunculatus* (Cnidaria), have been detected by PCR and could act as vectors for the parasite (Audemard *et al.,* 2002; Carrasco *et al.,* 2007).

2.2.7. Known or suspected wild aquatic animal carriers

Wild populations of flat oysters, *O. edulis,* and mussels, *Mytilus edulis* and *M. galloprovincialis,* are infected by *M. refringens* and might not exhibit clinical signs or mortality.

Marteilia refringens has been reported in wild *Solen marginatus* (López-Flores *et al*., 2008a), *Chamelea gallina* (López-Flores *et al*., 2008b), *Xenostrobus securis* (Pascual *et al*., 2010) and *Ostrea stentina* without a clear clinical impact of the parasite on these host species.

Other bivalve species have been listed as potential susceptible species for *M. refringens* and may thus play as carriers.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Transmission of *M. refringens* occurs, probably via an intermediate host (Audemard *et al.,* 2002; Carrasco *et al.,* 2008b). The parasite could be experimentally transmitted from the *O. edulis* and *M. galloprovincialis* to the copepod *Paracartia grani* (Audemard *et al.,* 2002; Carrasco *et al.,* 2008b). Transmission from *P. grani* to *O. edulis* or *M. galloprovincialis* has not been demonstrated experimentally (Audemard *et al.,* 2002; Carrasco *et al*., 2008b). In oysters, the early stages of disease occur in the stomach, palps and even gill epithelia. It is thought that initial infection occurs via feeding

currents. In mussels, the early stages have been observed in the epithelium of the gills, mantle, stomach and primary digestive tubules (Carrasco *et al.,* 2008a).

2.3.2. Prevalence

Prevalence is highly variable – up to 98% in *O. edulis*. Higher prevalence is expected depending on farming practices and in areas that have had more than 1 year of exposure to infection (Berthe *et al*., 2004; Grizel, 1985).

2.3.3. Geographical distribution

Reported in Albania, Croatia, France, Greece, Italy, Morocco, Portugal, Spain, Sweden, Tunisia, and the United Kingdom.

2.3.4. Mortality and morbidity

Infection is lethal for oysters: a 50–90% mortality rate is usually reported during summer and autumn, and is associated with sporulation of the parasite (Grizel, 1985; Grizel *et al*., 1974). Similarly, morbidity is higher during warmer periods. Mussels are less affected by infection but mortalities up to 40% were reported in impacted areas (Berthe *et al*., 2004; Villalba *et al*., 1993b) and naïve mussels presented 100% mortality after being cultured for 6 months in an infected area (Thébault *et al.,* 1999).

2.3.5. Environmental factors

The threshold temperature for parasite sporulation and transmission is 17°C. This temperature is common in estuaries or bays where prevalence is usually higher in the upper parts of the water column (Audemard *et al.,* 2001; Berthe *et al*., 2004; Carrasco *et al.,* 2007; Grizel, 1985). Infection with *M. refringens* is seldom observed in open sea waters (Grizel, 1985). High salinity and water renewal could be detrimental to *M. refringens* development and transmission, although these parameters appear to be less significant than temperature (Audemard *et al.,* 2001).

2.4. Control and prevention

2.4.1. Vaccination

None.

2.4.2. Chemotherapy

None.

2.4.3. Immunostimulation

None.

2.4.4. Resistance breeding

None.

2.4.5. Restocking with resistant species

Attempts have been made in Europe with different species of the genus *Ostrea,* but they have all demonstrated susceptibility (Grizel, 1985). Naïve stocks of *Ostrea edulis* and *Mytilus edulis* are highly susceptible to the infection. Although some *M. refringens* primary stages were observed in *Crassostrea gigas* (Berthe *et al*., 2004), this species appears resistant to infection with the parasite.

2.4.6. Blocking agents

None.

2.4.7. Disinfection of eggs and larvae

No data available.

2.4.8. General husbandry practices

Stocking at low density or in association with resistant mollusc species, such has *Crassostrea gigas,* has been shown to be effective (Grizel, 1985).

3. Sampling

3.1. Selection of individual specimens

Gaping or freshly dead individuals (2 or more years old) should be sampled by priority, to increase the chances of finding infected oysters. For histology, only live (including moribund) oysters or mussels should be sampled.

Sampling of flat oysters and mussels should be organised once a year when prevalence is known to be at a maximum. When such data are not available in a particular ecosystem, sampling should preferably be carried out when temperature reaches the yearly maximum (Audemard *et al.,* 2001; Carrasco *et al.,* 2007).

3.2. Preservation of samples for submission

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 might be relevant, but its impact on diagnostic tool performance has not been evaluated.

3.4. Best organs or tissues

A 3–5 mm thick section of tissues including gills and digestive mass is used for diagnostic of *M. refringens* by histology. A piece of digestive gland is preferred for some tests, including imprints and PCR.

3.5. Samples/tissues that are not suitable

Tissues other than gills and digestive mass are not suitable.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Clinical signs include dead or gaping molluscs, as weak animals are particularly susceptible to any additional stress (Grizel, 1985; Grizel *et al*., 1974). These clinical signs are not specific to infection with *M. refringens* and could be indicative of other infections.

4.1.2. Behavioural changes

Gaping.

4.2. Clinical methods

4.2.1. Gross pathology

Pale digestive gland, thin watery flesh, mantle retraction and reduced growth rate were reported for infected flat oysters (Berthe *et al*., 2004; Grizel, 1985; Grizel *et al*., 1974), although these gross signs are not specific to infection with *M. refringens*. Reduced growth rate and inhibition of gonad development were reported for infected mussels (Villalba *et al*., 1993a).

4.2.2. Clinical chemistry

None available.

4.2.3. Microscopic pathology

The digestive gland, in which *M. refringens* and other *Marteilia* species occur, is a site of intracellular food digestion and one of the main sites for storage of metabolic reserves (Berthe *et al*., 2004). In heavy infections, *M. refringens* significantly reduces absorption of organic matter (Robledo *et al*., 1995b). Severe infections may also cause loss of condition as a consequence of reduced energy acquisition. Furthermore, the parasite may interfere directly with host feeding and absorption simply by its physical presence. Development of adipo-granular storage cells in the mantle of *Mytilus galloprovincialis* was shown to be inhibited in the presence of *M. refringens* (Villalba *et al*., 1993b). Apparently, *M. refringens* also interferes with glycogen storage in *O. edulis* (Robert *et al.,* 1991).

Marteilia sydneyi is slightly different from *M. refringens*. In light and transmission electron microscopy, recognition criteria are based on the presence or absence of striated inclusions in the sporont primordia and concentric membranes surrounding mature spores, the number of sporangial primordia in plasmodium and the number of spores in sporangium.

4.2.4. Wet mounts

In advanced infection, mature sporangia with refringent granules can be observed in wet mounts from gaping oysters/mussels or freshly dead oysters/mussels or faeces of live oysters/mussels.

4.2.5. Smears

In advanced infection, parasites ranging in size up to 30–40 μm can be observed in digestive gland imprints from gaping oysters/mussels or freshly dead oysters/mussels..

4.2.6. Electron microscopy/cytopathology

In advanced infection, different parasite stages can be observed in the epithelia of the digestive tract.

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

In advanced infection, wet mounts are prepared.

Samples to be taken: gaping oysters/mussels or freshly dead oysters/mussels or faeces of live oysters/mussels.

Technical procedure: squash a piece of digestive gland or faeces on a glass slide. Observations are then made at ×400 and can potentially show refringent granules in mature sporangia.

Positive/negative controls: none.

Levels of validation:

- *Specificity and sensitivity:* unknown but presumably low;
- *Gold standard:* not validated against histology.

Interpretation of results:

- A positive result is the presence of large (20–30 μm) spherical bodies containing thick wall structures;
- In susceptible species, within the known geographical range of infection with *M. refringens*, a positive result is indicative of infection with *M. refringens;*

- In other species, or outside the known geographical range of infection with *M. refringens*, a positive result is indicative of infection with a *Marteilia* species that needs to be confirmed by the WOAH Reference Laboratory.

Availability of commercial tests: no kits available commercially.

4.3.1.1.2. Imprints

In advanced infection, digestive gland imprints are prepared.

Samples to be taken: gaping oysters/mussels or freshly dead oysters/mussels.

Technical procedure: after drying tissues on absorbent paper, several imprints are made on a glass slide. Slides are air-dried, fixed in methanol or in absolute ethanol and stained using a commercially available blood-staining kit, in accordance with the manufacturer's instructions. After rinsing in tap water and drying, the slides are mounted with a cover-slip using an appropriate synthetic resin. Slides are observed first at ×200 magnification and then under oil immersion at ×1000 magnification.

Positive controls: recommended and available from the WOAH Reference Laboratory.

Levels of validation:

- *Specificity and sensitivity:* unknown. As infection may be focal and also because infection targets different tissues in the early and late stages, imprints might miss early and low infection levels.
- *Gold standard:* not validated against histology.

Interpretation of results:

- A positive result is the observation of cells ranging in size up to 30–40 μm. The cytoplasm stains basophilic, whereas the nucleus stains eosinophilic. Pale halos around large, strongly stained (refringent) granules and, in larger cells, cell within cell arrangements are observed (Berthe *et al*., 2000; Berthe *et al*., 2004; Grizel *et al*., 1974).
- In susceptible species, within the known geographical range of infection with *M. refringens*, a positive result is strongly indicative of infection with *M. refringens.*
- In other species, or outside the known geographical range of infection with *M. refringens*, a positive result is indicative of infection with a *Marteilia* species that needs to be confirmed by the WOAH Reference Laboratory.

Availability of commercial tests: commercially available quick-staining kits include Difquick®[1]/ Hemacolor®.

4.3.1.1.3. Fixed sections

4.3.1.1.3.1. Histology

Samples to be taken: live oysters/mussels.

Technical procedure: sections of tissue that include gills, digestive gland, mantle, and gonad should be fixed for 24 hours in Davidson's fixative followed by normal processing for paraffin histology and staining, for example with haematoxylin and eosin. Observations are made at increasing magnifications up to ×1000.

Positive/negative controls: recommended and available from the WOAH Reference Laboratory.

1 Reference to specific commercial products as examples does not imply their endorsement by WOAH. This applies to all commercial products referred to in this *Aquatic Manual*.

Levels of validation:

- *Specificity and sensitivity:* Values of sensitivity and specificity for histology were estimated at 70% and 99%, respectively (Thébault *et al*., 2005).
- *Gold standard:* histology is the gold standard, and *in situ* hybridisation is co-validated with histology.

Interpretation of results:

- A positive result is the observation of cells ranging in size from 4 up to 40 μm. Young plasmodia (uninucleate) are mainly found in the epithelium of labial palps and stomach. Sporulation involves divisions of cells within cells and takes place in the digestive gland tubules and ducts. Refringent granules appear in the course of sporulation, but are not observed in early stages. In late phases of infection, sporangia are observed free in the lumen of the digestive tract. The cytoplasm stains basophilic, whereas the nucleus stains eosinophilic. The granules can range from deep orange to deep red;

  Marteilia refringens is slightly different from *M. sydneyi*. Recognition criteria are based on a lack of striated inclusions in the sporont primordia of *M. sydneyi*, formation of eight to sixteen sporangial primordia in each plasmodium, instead of eight for *M. refringens*, occurrence of two spores in each sporangium, rather than four in *M. refringens*, and the presence of a heavy layer of concentric membranes surrounding mature *M. sydneyi* spores.
- In susceptible species, within the known geographical range of infection with *M. refringens*, a positive result is conclusive for infection with *M. refringens;*
- In other species, or outside the known geographical range of infection with *M. refringens*, a positive result, especially with refringent granules being observed, is indicative of infection with a *Marteilia* species that needs to be confirmed by the WOAH Reference Laboratory.

Availability of commercial tests: no commercially available tests.

4.3.1.1.3.2. Transmission electron microscopy

Technical procedure: a small sized piece of tissue (1–2 mm) should be fixed in 3% glutaraldehyde (in 0.22 μm filtered sea water [FSW]) for 1 hour, washed three times in FSW, fixed in 1% osmic acid and washed twice again in FSW. After dehydration in successive baths of ethanol, and two baths of propylene oxide, samples should be progressively impregnated and embedded in Epon. After polymerisation at 60°C, blocks should be cut firstly at 0.5–1 μm for quality control and then at 80–100 nm for examination under an electron microscope. Ultrathin sections are placed on mesh copper grids and counterstained using uranyl acetate and lead citrate.

Positive controls: none.

Levels of validation:

- *Specificity and sensitivity:* Transmission electron microscopy (TEM) like histology permits the differentiation of *Marteilia refringens* from *M. sydneyi*. Recognition criteria are the same as those presented in section 4.3.1.1.3.1 Histology.

Interpretation of results:

- A positive result is the presence of parasites within the epithelia of the digestive gland or the stomach. Different parasite stages can be observed (Longshaw *et al*., 2001). The primary cell presents a single secondary cell within it. Secondary cells result from a series of divisions and include eight presporangia. Tertiary cells contain these eight presporangia that have divided containing four-spore primordia. Spore primordia cleave internally to produce mature spores. Mature spores consist of three sporoplasms, one inside the other, the outermost one containing haplosporosomes.

Availability of commercial tests: no commercially available tests.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Not available.

4.3.1.2.2. Antibody-based antigen detection methods

Not currently available or used for diagnostic purposes but monoclonal antibodies have been developed and published (Berthe *et al*., 2004).These antibodies did not cross react against *M. sydneyi*.

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. Polymerase chain reaction (*PCR*)

*PCR protocols have been developed and published (*Berthe *et al*., 2000*;* Le Roux *et al*., 1999; 2001; López-Flores *et al*., 2004*).*

PCR primers that target the ITS1 (internal transcribed spacer) region (Le Roux *et al*., 2001) are recommended as they are able to amplify only *M. refringens.* However some primers targeting the small subunit (SSU) of the rRNA gene complex are also available and allow *M. refringens* and *M. sydneyi* to be amplified (Grizel *et al*., 1974; Le Roux *et al*., 1999). In addition, a nested PCR assay was developed using primers targeting the rDNA intergene spacer (López-Flores *et al*., 2004). It has been tested only with *M. refringens*. This assay was demonstrated to be more sensitive than ITS1 PCR assay but needs to be tested more thoroughly for specificity.

Samples to be taken: live oysters/mussels or freshly dead oysters/mussels.

Technical procedure: tissue samples are placed in 95–100% ethanol or frozen until DNA is extracted DNA extraction is accomplished by proteinase K digestion overnight at 50–55°C, and phenol–chloroform extraction with ethanol precipitation or the spin-column methodology using commercially available kits (e.g. Qiagen). PCR is carried out in a 50 µl volume. PCR mixtures contain buffer (500 mM KCl, 100 mM Tris/HCl [pH 9.0 at 25°C] and 1% Triton® X-100), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 µM forward and reverse primers, 0.02 units μl^{-1} Taq DNA polymerase, and 10–100 ng of extracted DNA. After denaturation of DNA at 94°C for 5 minutes, 30 cycles are run as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 1 minute per kilo-base pair. A final elongation step of 10 minutes at 72°C is performed. For the detection of *M. refringens*, PCR is performed with primers that target the ITS1 region (5'-CCG-CAC-ACG-TTC-TTC-ACT-CC-3' and 5'-CTC-GCG-AGT-TTC-GAC-AGA-CG-3') (Le Roux *et al*., 2001).

Positive/negative controls: these are compulsory. Positive controls are: 1) PCR with specific primers to genomic DNA from a highly infected host or DNA from purified parasites; 2) nonspecific amplification (actin, SSU, etc.). Negative controls are: 3) no target DNA reactions; 4) PCR with specific primers on genomic DNA from non-infected hosts. Positive controls are available on request from the WOAH Reference Laboratory.

Levels of validation:

- *Specificity and sensitivity:* unknown values. No cross-reaction has occurred with tested samples and specificity is considered very high (Kleeman *et al*., 2002; Le Roux *et al*., 1999). This PCR technique is expected to detect *M. refringens*. As infection may be focal and also because infection targets different tissues in the early and late stages, the sensitivity of PCR detection may be lower than the expected theoretical PCR performance.
- *Gold standard:* not validated against histology.

Interpretation of results:

- A positive result is positive PCR amplification at the expected size, with all negative controls being negative and all positive controls being positive.

- In susceptible species, within the known geographical range of infection with *M. refringens*, a positive PCR result, associated with a positive result by means of histology or imprints, is confirmatory for infection with *M. refringens*.
- In other species, or outside the known geographical range of infection with *M. refringens*, a positive PCR result, associated with a positive result by means of histology or imprints, is strongly indicative of infection with *M. refringens,* but PCR product sequencing is necessary before confirmatory diagnosis.

Availability of commercial tests: no commercially available tests.

4.3.1.2.3.2. In-situ *hybridisation* (*ISH*)

ISH protocols have been developed and published (Berthe *et al*., 2000; Le Roux *et al*., 1999).

Probe that targets the SSU of the rRNA gene complex is recommended because it has been validated against histology (Le Roux *et al*., 1999; Thébault *et al*., 2004). However this probe was shown to cross react with *Marteilia sydneyi* and *Marteilioïdes chungmuensis* (Kleeman *et al*., 2002). In addition, an ISH assay was developed using a probe targeting the rDNA intergene spacer (López-Flores *et al*., 2008a; 2008b). This assay was demonstrated to be more specific than the SSU ISH assay but needs to be thoroughly validated.

Samples to be taken: live/mussels oysters or gaping oysters/mussels.

Technical procedure: for ISH, molluscs are fixed in Davidson's fixative for approximately 24 hours and then embedded in paraffin. Sections of 5 μm are cut and placed on aminoalkylsilane-coated slides, which are then baked overnight in an oven at 40°C. The sections are dewaxed by immersing in xylene or equivalent for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections are then rehydrated by immersion in an ethanol series. The sections are treated with proteinase K (100 μg ml^{-1}) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 30 minutes. Slides are dehydrated by immersion in an ethanol series and then air dried. Sections are incubated with 100 μl of hybridisation buffer (4 × SSC [standard saline citrate], 50% formamide, 1 × Denhardt's solution, 250 μg ml^{-1} yeast tRNA, 10% dextran sulphate) containing 10 ng (1 μl of the PCR reaction prepared like described above using primers CCG-GTG-CCA-GGT-ATA-TCT-CG and TTC-GGG-TGG-TCT-TGA-AAG-GC) of the digoxigenin-labelled probe (Le Roux *et al.*, 1999). Sections are covered with *in-situ* plastic cover-slips and placed on a heating block at 95°C for 5 minutes. Slides are then cooled on ice for 1 minute before overnight hybridisation at 42°C in a humid chamber. Sections are washed twice for 5 minutes in 2 × SSC at room temperature, and once for 10 minutes in 0.4 × SSC at 42°C. The detection steps are performed according to the manufacturer's instructions. The slides are then rinsed in sterile distilled water (dH_2O). The sections are counter-stained with Bismarck Brown Yellow, rinsed in dH_2O, and cover-slips are applied using an aqueous mounting medium.

Positive/negative controls: compulsory. Positive controls are: 1) ISH on infected host; 2) nonspecific ISH (SSU rDNA) on samples. Negative controls are: 3) no probe ISH reactions; 4) ISH on non-infected hosts. Positive controls are available on request from the WOAH Reference Laboratory.

Levels of validation:

- *Specificity and sensitivity:* 90% and 99%, respectively, in the case of ISH (Thébault *et al*., 2005). This ISH protocol is expected to detect *M. refringens;* however this probe was shown to cross react with *M. sydneyi* and *Marteilioïdes chungmuensis* (Kleeman *et al*., 2002).
- *Gold standard:* co-validated with histology.

Interpretation of results:

- A positive result is demonstrated by the purple-black labelling of *M. refringens* cells within known target tissues, with all negative controls being negative and all positive controls being positive.
- In susceptible species, within the known geographical range of infection with *M. refringens*, a positive ISH result is confirmatory of infection with *M. refringens.*

- In other species, or outside the known geographical range of infection with *M. refringens*, a positive ISH result needs further molecular characterisation in order to determine the parasite species. Such a case should, however, be referred to the appropriate WOAH Reference Laboratory.

Availability of test: the probe can be obtained from the appropriate WOAH Reference Laboratory.

4.3.1.2.3.3. Sequencing

Sequencing is recommended as one of the final steps for confirmatory diagnosis. Targeted regions are SSU rDNA and ITS1. Although the sequences are available in the public gene banks, it is recommended to refer such cases to the appropriate WOAH Reference Laboratory.

4.3.1.2.4. Agent purification

Agent purification is not currently used for diagnostic purposes, but purification protocols have been developed and published (Miahle *et al.,* 1985; Robledo *et al*., 1995a).

4.3.2. Serological methods

None applicable.

5. Rating of tests against purpose of use

As an example, the methods currently available for targeted surveillance and diagnosis of infection with *M. refringens* 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
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	c	d
Wet mounts	d	d	c	c	c	d
Imprints	d	d	b	b	a	c
Histopathology	d	d	a	a	b	c
***In-situ* DNA probes**	d	d	d	d	d	b
PCR	a	a	a	a	a	a
Sequence	d	d	d	d	d	a

PLs = postlarvae; PCR = polymerase chain reaction.

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

Prescribed methods for targeted surveillance to declare freedom from infection, as outlined in the *Aquatic Code* are: tissue imprints (digestive gland), histology or PCR.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Any positive result obtained by any diagnostic technique should be considered suspect.

7.2. Definition of confirmed case

In known susceptible species and within the known geographical range, a confirmed case of *M. refringens* is a positive result by tissue imprints or histology combined with ISH or PCR.

In other host species, or outside the known range of *M. refringens*, confirmation by sequencing and description by transmission electron microscopy are recommended.

8. References

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NB: There is a WOAH Reference Laboratory for infection with *Marteilia refringens*
(please consult the WOAH web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on infection with *Marteilia refringens*

NB: FIRST ADOPTED IN 1995 AS MARTEILIOSIS. MOST RECENT UPDATES ADOPTED IN 2023 (SECTIONS 2.2.1 AND 2.2.2).