**Annex 33. Item 8.2.3. – Chapter 2.4.4. Infection with *Marteilia refringens***

**USA COMMENTS IN RED FONT**

Chapter 2.4.4.

infection with *Marteilia refringens*

1. Scope

Infection with *Marteilia refringens* means infection with the pathogenic agent *M. refringens* (including O and M types) of the Family *Marteiliidae.*

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

*Marteilia refringens* is a protozoan parasite of the Family *Marteiliidae* (Cavalier-Smith & Chao, 2003; Feist *et al.*, 2009) infecting the digestive system of several bivalve species and inducing physiological disorders and eventual death of the animal (Alderman, 1979; Grizel *et al*., 1974). Two types of *M. refringens* (Grizel *et al*., 1974), types O and M, were defined by Le Roux *et al.* (2001). Although more recent results suggest that *M. refringens* should be distinguished from *M. pararefringens* (previously *M. maurini* or *M. refringens* type M) (Kerr *et al.*, 2018), a larger set of samples is required to properly define both species and most available data in the literature do not allow differentiation of *M. refringens* type O (= *M. refringens* in Kerr *et al.*, 2018) or *M. refringens* type M (= *M. pararefringens* in Kerr *et al.*, 2018) to be made.

2.1.2. Survival and stability in processed or stored samples

No information available

2.1.3. Survival and stability outside the host

After its release from the European flat oyster (*Ostrea edulis*), *M. refringens* can survive at least 20 days in seawater and faeces. Parasite survival seems improved in faeces compared with seawater (Mérou *et al.*, 2022).

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 *M. 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~~*~~).~~

**RATIONALE:** The U.S. does not support the listing of species for which there is "incomplete susceptibility" as to date this is subjective since WOAH has not published any criteria for determining that a species meets "incomplete susceptibility" criteria. If a given specifies does not meet the WOAH criteria for listing as susceptible, then it should not be included in the WOAH chapter.

The inclusion of species which do not meet the criteria for listing as susceptible, may have adverse trade impacts due to member countries feeling like they should be regulating the movement of these species to protect their health status, when in fact there is insufficient scientific rational to do so and this could be seen as a non-scientific barrier to trade (which is against WTO standards).

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

*Marteilia refringens* usually causes clinical infection in the European flat oyster, *O. edulis* (Berthe *et al.*, 2004; Grizel *et al.*, 1974). In flat oysters and mussels, prevalence and infection intensity are generally higher in individuals 2 years old or older (Audemard *et al.*, 2001; Villalba *et al.*, 1993b).

2.2.4. Distribution of the pathogen in the host

*Marteilia refringens* infects the digestive tract. Young plasmodia are mainly found in the epithelium of labial palps, oesophagus 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; Mérou *et al.*, 2022).

2.2.5. Aquatic animal reservoirs of infection

Infected flat oysters, *O. edulis,* and mussels, *Mytilus edulis* and *M. galloprovincialis,* might not exhibit clinical signs or mortality, however they can release parasite sporangiospores (Arzul *et al.,* 2014; Mérou *et al.,* 2023).

2.2.6. Vectors

None known.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

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).

Prevalence is highly variable – up to 98% in *O. edulis*. Higher prevalence is expected depending on farming practices and in areas where potential hosts have had more than 1 year of exposure to infection (Berthe *et al*., 2004; Grizel, 1985). Prevalence usually peaks in summer whereas the parasite is usually absent or found at lower infection intensity in winter and early spring (Audemard *et al.*, 2001; Mérou *et al.*, 2023). An additional prevalence peak in spring has been reported in several studies (Arzul *et al.*, 2014; Boyer *et al.*, 2013; Carrasco *et al.*, 2007; Mérou *et al.*, 2023).

2.3.2. Clinical signs, including behavioural changes

Clinical signs include dead or gaping molluscs (Grizel, 1985; Grizel *et al*., 1974) but are not specific for infection with *M. refringens* and could be indicative of other infections.

2.3.3 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 for infection with *M. refringens*. Reduced growth rate and inhibition of gonad development were reported for infected mussels (Villalba *et al*., 1993a).

2.3.4. Modes of transmission and life cycle

Horizontal 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 *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 oesophagus, 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).

The life cycle of *M. refringens* is suspected to be indirect and may include *P. grani* (Audemard *et al.,* 2001; 2002), at least in pond systems. Other species (see Sections 2.2.5 and 2.2.6) might be involved as reservoirs or vectors in the *M. refringens* life cycle but their role has not been demonstrated).

The detection of *M. refringens* DNA in plankton, particularly nanoplankton, and in the benthos, suggests their involvement in the parasite life-cycle including transmission and storage or possible overwintering, respectively (Mérou *et al.*, 2023).

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).

Parasite DNA detection in pelagic compartments was found higher when temperature, salinity and cholorophyll-a were higher (Mérou *et al.*, 2023).

2.3.6. Geographical distribution

Reported in Europe and North Africa.

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

None.

2.4.3. Immunostimulation

None.

2.4.4. Breeding resistant strains

None.

2.4.5. Inactivation methods

No data available.

2.4.6. Disinfection of eggs and larvae

No data available.

2.4.7. General husbandry

Stocking at low density or in association with resistant mollusc species, such as *Crassostrea gigas,* has been shown to be effective (Grizel, 1985). Stocking bivalves in deep zones exposed to currents seems to limit the transmission of the parasite. Considering the possible presence of the parasite in the sediment (Mérou *et al.*, 2023), maintaining bivalves at distance from the bottom should limit the number of infected animals.

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 or freshly dead individuals (2 or more years old) of species referred to in Section 2.2.1., should be sampled preferentially, to increase the chances of finding infected bivalves. For histology, only live (including moribund) bivalves should be sampled.

Sampling of bivalves should be organised 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. Selection of organs or tissues

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

3.3. Samples or tissues not suitable for pathogen detection

Tissues other than gills and digestive mass are not suitable.

3.4. Non-lethal sampling

Examination of fresh samples of faeces collected from potentially infected bivalves using light microscopy is possible although this approach has not been validated (See Section 4.1)

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

The success of pathogen isolation and results of bioassay depend strongly on the quality of samples (time since collection and time in storage). 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 histological 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.

Performances of diagnostic methods applied on pools have not been evaluated.

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 |  |  |  |  |  | + | + | NA |  |  |  |  |
| Tissue imprints |  | ++ | ++ | NA |  | +++ | +++ | NA |  |  |  |  |
| Histopathology |  | ++ | ++ | 2 |  | +++ | +++ | NA |  |  |  |  |
| Transmission electron microscopy |  |  |  |  |  |  |  |  | + | ++ | ++ | NA |
| Real-time PCR | +++ | +++ | +++ | 3 | +++ | +++ | +++ | NA | +++ | +++ | +++ | NA |
| Conventional PCR | ++ | ++ | ++ | 2 | +++ | +++ | +++ | NA |  |  |  |  |
| Conventional PCR followed by amplicon sequencing |  |  |  |  |  |  |  |  | +++ | +++ | +++ | NA |
| *In-situ* hybridisation |  |  |  |  |  |  |  |  | + | +++ | +++ | NA |
| Bioassay |  |  |  |  |  |  |  |  |  |  |  |  |
| LAMP |  |  |  |  |  |  |  |  |  |  |  |  |
| Ab-ELISA |  |  |  |  |  |  |  |  |  |  |  |  |
| Ag-ELISA |  |  |  |  |  |  |  |  |  |  |  |  |
| Other antigen detection methods3 |  |  |  |  |  |  |  |  |  |  |  |  |
| Other methods3 |  |  |  |  |  |  |  |  |  |  |  |  |

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.   
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. Wet mounts

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

Squash a piece of digestive gland on a glass slide. Observations are then made at ×400 magnification and can potentially show refringent granules in mature sporangia.

*Marteilia* species are indicated by the presence of large (9–30 µm) spherical bodies containing thick wall structures.

4.2. Imprints

In moderate and advanced infections, digestive gland imprints are prepared.

Samples to be taken consist of fresh, gaping, or freshly dead bivalves.

*A*fter drying tissues on absorbent paper, several imprints are made on a glass slide. Slides are air-dried, fixed and stained using a commercially available blood-staining kit, in accordance with the manufacturer’s instructions; fixation can be done using methanol or absolute ethanol. 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.

The observation of cells with a range in size of 5–8 µm diameter in the early stages of development and up to 30–40 µm during sporulation, may indicate infection with *Marteilia refringens.* 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. In advanced stages, eight secondary cells can be observed in the primary cells and four spores in each secondary cell (Berthe *et al*., 2000; 2004; Grizel *et al*., 1974).

4.3. Histopathology

Samples to be taken consist of live or moribund bivalves.

*S*ections of tissues that include gills, digestive gland, mantle and gonad 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 ×1000.

*Specificity and sensitivity:* values of diagnostic sensitivity and specificity for histology were estimated at 70% and 99%, respectively (Thébault *et al*., 2005).

The observation of cells ranging in size from 4 to 40 µm may be indicative of infection with *Marteilia refringens*. Young stages (uninucleated primary cells) are mainly found in the apical part of the epithelium of labial palps, stomach and sometimes in the digestive tubules. Sporulation involves divisions of cells within cells and generally takes place in the digestive gland tubules and ducts. Refringent granules appear during sporulation but are not observed in early stages. The cytoplasm stains basophilic, whereas the nucleus stains eosinophilic. The granules can range from deep orange to deep red; *M. refringens* can sometimes be observed in other organs including gill and mantle connective tissues (Carrasco *et al.*, 2015; Grizel *et al.*, 1974).

*Marteilia refringens* is slightly different from other *Marteilia* species including *M. sydneyi* or *M. octospora.* Recognition criteria are mainly based on the number of secondary and tertiary cells (respectively 8 and 4 for *M. refringens*). Although *M. christenseni* and *Eomarteilia granula* display the same number of secondary and tertiary cells as *M. refringens,* they infect different host species in different geographic zones.

4.4. Transmission electron microscopy

A small-sized piece of digestive gland (1–2 mm) should be fixed in an appropriate fixative for at least 1 hour and then processed as described in Section B.5.4 *Transmission electron microscopy methods* of Chapter 2.4.0 *General information* (diseases of molluscs).

The presence of parasites within the epithelia of the digestive gland or the stomach may be indicative of infection with *Marteilia refringens*. Different parasite stages can be observed (Grizel *et al.,* 1974; Longshaw *et al*., 2001). The first stage (= primary cell) is uninucleated but is often observed presenting a single secondary cell within it. Secondary cells result from a series of divisions within the primary cells and include eight presporangia. These presporangia (=secondary cells) divide and contain four-spore primordia (= tertiary cells). Spore primordia cleave internally to produce mature spores. Mature spores consist of three sporoplasms, one inside the other, the outermost one containing haplosporosomes.

4.5. Nucleic acid amplification

Samples to be taken consist of tissues of digestive gland and gills from live or freshly dead molluscs.

PCR assays should always include the controls specified in Section B.5.5 *Molecular methods* of Chapter 2.4.0 *General information* (diseases of molluscs). Molluscs are known to potentially contain substances that can inhibit PCR reactions. It is recommended to check for the presence of PCR inhibitors in DNA extracts to avoid false negative results. In case PCR inhibitors are present, DNA samples can be diluted prior to PCR analyses (a 1/10 dilution resolves most cases of PCR inhibition).

*Extraction of nucleic acids*

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.

4.5.1. Real-time PCR

Two multiplex real-time PCR assays targeting the ITS (internal transcribed spacer) gene have been developed for the specific detection and discrimination of *M. refringens* type O and type M (Carrasco *et al*., 2017; EURL, 2023).

Additionally, a multiplex real-time PCR assay targeting the 18S gene allows the concomitant detection of *M. refringens* and *Bonamia* spp. parasites (Canier *et al.*, 2020). However, validation tests showed that this PCR assay is less specific and also amplifies *M. cochillia* and to a lesser extent *M. sydneyi*.

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

|  |  |  |  |
| --- | --- | --- | --- |
| Pathogen/ target gene | Primer/probe (5’–3’) | Concentration | Cycling parameters(a) |
| Method 1: Carrasco *et al.* (2017); GenBank Accession No.: MH304865.1 | | | |
| *M. refringens* types O and M  ITS | Fwd Mare-F: YCA-GGC-GAG-TGC-TCT-CGT-T Rev Mare-R: TGA-TCT-GAT-ATT-ATT-CAG-CTG-TTC-GA  Probe Mare-O: CCT-TTC-CCC-GAC-GGC (VIC MGB-NFQ) Probe MareM: GCT-TGC-CCT-ACG-GCC (FAM MGB-NFQ) | 400 nM 400 nM  80 nM 80 nM | 50 cycles of: 95°C/3 sec and 60°C/30 sec |
| Method 2: EURL (2023); GenBank Accession No.: MH304863.1 | | | |
| *M. refringens* types O and MITS | Fwd TaqMar-F: GTG-TTC-GGC-ACG-GGT-AGT Rev TaqMar-R: TGA-TCT-GAT-ATT-ATT-CAG-CTG-TTC-G  TaqProb-O: GCC-CTT-TCC-CCG-ACG-GCC-G (FAM-BHQ-1) TaqProb-M: GCG-CTT-GCC-CTA-CGG-CCG-TGC (HEX-BHQ-1) | 100 nM 300 nM  250 nM 250 nM | 40 cycles of: 95°C/30 sec and 60°C/1 min |
| Method 3: Canier *et al.* (2020); GenBank Accession No.: MH342044.1 | | | |
| *M. refringens* Also amplifies *M. cochillia* and *M. sydneyi*  18S | Fwd Mar\_18S\_F: ACG-ATC-AAA-GTG-AGC-TCG-TG Rev Mar\_18S\_R: CAG-TTC-CCT-CAC-CCC-TGA-T Probe Mar\_18S\_IN: GCA-TGG-AAT-CGT-GGA-ACG-GG (FAM-BHQ-1) | 400 nM 400 nM 300 nM | 40 cycles of: 95°C/15 sec and 60°C/1 min |

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

4.5.2. Conventional PCR

PCR primers are available that target the ITS1 (internal transcribed spacer) region (Le Roux *et al*., 2001), 18S gene (Le Roux *et al*., 1999) and the IGS (rDNA intergenic spacer) region (López-Flores *et al*., 2004).

***Primer sequences***

|  |  |  |  |
| --- | --- | --- | --- |
| Pathogen/ target gene | Primer (5’–3’) | Concentration | Cycling parameters(a) |
| Method 1: Le Roux *et al.* (2001); GenBank Accession No.: MH329403.1; amplicon size 412 bp | | | |
| *M. refringens* types M and O Also amplifies *M. cochillia* ITS-1 | Fwd Pr4 (M2A): CCG-CAC-ACG-TTC-TTC-ACT-CC Rev Pr5 (M3AS): CTC-GCG-AGT-TTC-GAC-AGA-CG | 1000 nM 1000 nM | 30 cycles of: 95°C/1 min and 55°C/1 min and 72°/1 min |
| Method 2: Lopez-Flores *et al.* (2004) (nested PCR) ; GenBank Accession No.: MH356753.1; amplicon size [525bp & 358 bp] | | | |
| *M. refringens* types M and O Also amplifies *M. cochillia* and possibly other species IGS | PCR1 Fwd MT1: GCC-AAA-GAC-ACG-CCT-CTA-C  Rev MT2: AGC-CTT-GAT-CAC-ACG-CTTT  PCR2 Fwd MT-1B: CGC-CAC-TAC-GAC-CGT-AGC-CT Rev MT-2B: CGA-TCG-AGT-AAG-TGC-ATG-CA | 1000 nM 1000 nM   1000 nM  1000 nM | PCR 1 I30 cycles of: 95°C/1 min and 55°C/1 min and 72°/1 min  PCR2 25 cycles of: 95°C/30 sec and 60°C/30 sec and 72°/30 sec |
| Method 3: Le Roux *et al.* (1999); GenBank Accession No.: MH342044.1; amplicon size [266bp or 700 bp] | | | |
| *Marteilia* spp*.* amplifies *M. refringens* types M and O, *M. cochillia*, and possibly other species  18S | Fwd SS2: CCG-GTG-CCA-GGT-ATA-TCT-CG (Rev SAS1: TTC-GGG-TGG-TCT-TGA-AAG-GC) Or Rev SAS2: CGA-ACG-CAA-ATT-GCG-CAG-GG | 1000 nM 1000 nM  1000 nM | 30 cycles of: 95°C/1 min and 55°C/1 min and 72°/1 min |
| Note: according to the alignment of available sequences  Rev SAS1 primer sequence should be: TTC-GG-TGG-TCT-TGA-AAG-GC | | | |

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

4.5.3. Other nucleic acid amplification methods

A loop-mediated isothermal amplification (LAMP) assay for the detection of *M. refringens* has been developed, but is not validated (Xie *et al.*, 2012).

4.6. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis and purified by excision from this gel for sequence analysis. Obtained sequences are compared with published sequences.

Sequencing is recommended as one of the final steps for confirmatory diagnostic. Targeted regions are the SSU rDNA (except 18S PCR SS2/SAS1), ITS1 and IGS (intergenic spacer). Although sequences are available in the public gene banks, it is recommended to refer such cases to the appropriate WOAH Reference Laboratory.

4.7. *In-situ* hybridisation

Le Roux *et al.* (1999) developed an ISH genus-specific method targeting the 18S gene. This method allows the detection of all currently known *Marteilia* species. It has been validated against histology for the detection of *M. refringens* (Thébault *et al.*, 2005).

Two other ISH assays have been developed, one targeting the ITS1 (internal transcribed spacer) region (Le Roux *et al*., 2001) and the other targeting the IGS (intergenic spacer) region (Lopez-Flores *et al*., 2008a; 2008b). These assays allow the detection of *M. refringens* type O and type M.

Samples to be taken consist of live or gaping molluscs.

*Technical procedure:*

|  |  |  |  |
| --- | --- | --- | --- |
| Reference | Pathogen/target gene | ISH probe | Probe size |
| Le Roux *et al.* (1999) | *Marteilia* sp.  18S | Digoxigenin-labelled PCR product obtained with SS2/SAS1 primers | 266 bp |
| Le Roux *et al.* (2001) | *M. refringens* types M and O  ITS1 | Digoxigenin-labelled PCR product obtained with Pr4/Pr5 primers | 412 bp |
| Lopez-Flores *et al.* (2004) | *M. refringens* types M and O IGS | Digoxigenin-labelled PCR product obtained with MT-1B/MT-2B primers | 358 bp |

The first steps follow the recommendations described in Section B.5.5.4. of Chapter 2.4.0 *General information* (diseases of molluscs). For hybridisation, 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 approx. 10 ng (2 to 5µl µl of digoxigenin-labelled probe prepared by conventional PCR as described above (section 4.5.2; Le Roux *et al.*, 1999; 2001, Lopez-Flores *et al*., 2004; 2008a; 2008b). Sections are covered with *in-situ* plastic cover-slips and placed on a heating block at 94°C for 5 minutes. Slides are then cooled on ice for 1 to 5 minutes 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 with appropriate buffer. The sections are counter-stained with Bismarck Brown Yellow, rinsed in tap water, immersed in 95% and then 100% ethanol, 30 seconds for each, rinsed in Xylene (10–30 seconds), and cover-slips are applied using an appropriate mounting medium.

*Positive/negative controls:* inclusion of the following controls iscompulsory. 1) Infected host positive control; 2) non-specific ISH (18S) on samples as an internal positive control. 3) No probe ISH negative control; 4) Uninfected host negative control. Positive controls are available on request from the WOAH Reference Laboratory.

4.8. Immunohistochemistry

Not available.

4.9. Bioassay

Not available.

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

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

4.11. Other methods

None available.

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

Real-time PCR is recommended for targeted surveillance to declare freedom from infection with *M. refringens*.

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.

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

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link 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 *M. refringens* shall be suspected if at least one of the following criteria is met:

i) Positive result by a recommended molecular detection test

ii) Visual observation of the pathogen by microscopy

6.1.2. Definition of confirmed case in apparently healthy animals

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

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

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 *M. refringens* shall be suspected if at least one of the following criteria is met:

i) Positive result by wet mounts

ii) Positive result by tissue imprints

iii) Positive result by histopathology

iv) Positive result by real-time PCR

v) Positive result by conventional PCR

6.2.2. Definition of confirmed case in clinically affected animals

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

i) positive result by real-timePCR and conventional PCR followed by sequence analysis

ii) positive result by species-specific ISH and conventional PCR followed by sequence analysis

iii) Positive result of real-time PCR followed by species-specific ISH

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *M. refringens* are provided in Tables 6.3.1. (no data are currently available) and 6.3.2. This information can be used for the design of surveys for infection with *M. refringens*, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. 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 [under study]

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 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 ~~samples~~ animals used in the validation study.

**RATIONALE:** Edits clarify that the variable "n" actually stands for the number of animals included in the validation study, rath than the sample size recommendation which results from the study.

6.3.2. For surveillance of apparently healthy animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Histology | Surveillance | Field samples from France and The Netherlands, representative of 3 different levels of prevalence (free, mild, high) | Section of tissues including visceral mass | Flat oysters | 70% (200) | 99%  (200) | *In-situ* hybridisation (18S probe) Bayesian analyses | Thébault *et al.*, 2005 |
| *In-situ* hybridisation (18S probe) | Surveillance | Field samples from France and The Netherlands, representative of 3 different levels of prevalence (free, mild, high) | Section of tissues including visceral mass | Flat oysters | 90% (200) | 99% (200) | Histology Bayesian analyses | Thébault *et al.*, 2005 |
| Real-time PCR (Canier *et al.*, 2020) | Surveillance | Field samples from the 3 main producing areas in France, representative of 3 different levels of prevalence (free, low, high) | Gills and digestive gland tissues | Flat oysters | 87,2% (386) | 98,4% (386) | Conventional PCR (Le Roux *et al.*, 2001) Bayesian analyses | Canier *et al.*, 2020 |
| Conventional PCR (Le Roux *et al.*, 2001) | Surveillance | Field samples from the 3 main producing areas in France, representative of 3 different levels of prevalence (free, low, high) | Gills and digestive gland tissues | Flat oysters | 60.7% (386) | 99.9% (386) | Real-time PCR (Canier *et al.*, 2020) Bayesian analyses | Canier *et al.*, 2020 |

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

**RATIONALE:** Edits clarify that the variable "n" actually stands for the number of animals included in the validation study, rath than the sample size recommendation which results from the study.

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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 2012.

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