Annex 23. Item 8.3.2. – Chapter 2.4.3. ‘Infection with *Bonamia ostreae*’

Chapter 2.4.3.

infection with *Bonamia ostreae*

1. Scope

Infection with *Bonamia ostreae* means infection with the pathogenic agent *Bonamia ostreae* of the Family Haplosporiidae.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

*Bonamia ostreae* is a *Haplosporidian* protozoan parasite (Arzul & Carnegie, 2015; Carnegie & Cochennec-Laureau, 2004) infecting haemocytes of flat oysters, *Ostrea edulis,* and causing disease and mortality (Grizel, 1985).

2.1.2. Survival and stability in processed or stored samples

No data available.

2.1.3. Survival and stability outside the host

After its release from *Ostrea edulis*, *B. ostreae* can survive at least 4 days in seawater, however more than 90% of shed parasites are no longer detected after 2 days outside the oysters (Mérou *et al.,* 2020). Up to 58% of parasites isolated from highly infected oysters seem to survive after 1 week in seabed bore water at 15°C (Arzul *et al.,* 2009).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

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

| **Family** | **Scientific name** | **Common name** |
| --- | --- | --- |
| Ostreidae | *Magallana* (syn. *Crassostrea*) *ariakensis* | Ariake cupped oyster |
| *Ostrea chilensis* | Chilean flat oyster |
| *Ostrea edulis* | European flat oyster |

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 *B. ostreae* according to Chapter 1.5 of the *Aquatic Code* are:

| **Family** | **Scientific name** | **Common name** |
| --- | --- | --- |
| Ostreidae | *Ostrea puelchana s* | Argentinean flat oyster |

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** |
| --- | --- | --- |
| Actiniidae | *Actina equina* | beadlet anemone |
| Ascidiidae  | *Ascidiella aspersa* | European sea squirt |
| Ophiotrichidae | *Ophiothrix fragilis* | brittle star |
| Ostreidae | *Magallana* (syn. *Crassostrea*) gigas | Pacific cupped oyster |
| N/A | grouped zooplankton | zooplankton |

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

All ages of oysters appear susceptible to *B. ostreae* including larvae and spat (Arzul *et al.,* 2010), however, prevalence and infection intensity are generally higher in individuals of 2 years of age or more particularly post-spawning (Culloty & Mulcahy, 1996).

2.2.4. Distribution of the pathogen in the host

*Bonamia ostreae* is an intrahaemocytic protozoan (Comps *et al*., 1980; Pichot *et al*., 1979) but it can be observed extracellularly between epithelial or interstitial cells in the gills and stomach or in necrotic connective tissue areas. Intraepithelial localisation has also been reported in gills (Montes *et al*., 1994). The parasite was also reported in ovarian tissue (van Banning, 1990). Advanced infections become systemic. In larvae, the parasite was observed in the epithelium surrounding the visceral cavity (Arzul *et al.,* 2010).

2.2.5. Aquatic animal reservoirs of infection

Any susceptible species(see Section 2.2.1) and any specieswith incomplete evidence for susceptibility (see Section 2.2.2.) should be considered as potential reservoir. In addition, the observation of parasites presumably *B. ostreae* in *O. angasi* displayed in a zone infected with *B. ostreae*, suggests that this species could also be a reservoir for *B. ostreae* (Bougrier *et al.,* 1986)*.*

2.2.6. Vectors

The possible role of benthic macroinvertebrates and zooplankton in the life cycle of *B. ostreae* was investigated. The brittle star *Ophiothrix fragilis* was identified as a possible vector for the parasite (Lynch *et al.,* 2006).

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Infection of wild and cultured flat oysters is often lethal, and death usually occurs concurrently with the highest intensity of infection.

Prevalence is variable (from 0% to 80%) and is higher in individuals older than 2 years. The disease occurs and can be transmitted throughout the year, but there is a seasonal variation in infection with *B. ostreae,* with prevalence of infection increasing from autumn and showing a peak in late winter/early spring (Arzul *et al.,* 2006; Culloty & Mulcahy, 1996; Engelsma *et al.,* 2010; Grizel, 1985; Mérou *et al.,* 2023).

2.3.2. Clinical signs, including behavioural changes

Clinical signs include dead or gaping oysters.

2.3.3 Gross pathology

Gross pathology includes occasional yellow discoloration, extensive lesions including perforated ulcers in the connective tissues of the gills, mantle and digestive gland (Comps *et al*., 1980). These gross signs are not pathognomonic for infection with *B. ostreae* and most infected oysters appear normal.

2.3.4. Modes of transmission and life cycle

Direct transmission from host to host is possible. Transmission of the parasite directly from host to host by cohabitation or by inoculation of purified parasites has been demonstrated experimentally (Hervio *et al.,* 1995), suggesting that no intermediate host is needed. This is supported by the correlation between oyster density and prevalence of bonamiosis (Grizel, 1985).

The observation of parasites in the epithelium of palleal organs including gills suggests that the parasite enters into and is released from the oysters through these organs.

Moreover, the parasite was observed in larvae incubated in the pallial cavity of adult oysters suggesting possible transmission between these two age groups. Thus, larvae may contribute to the spread of the parasite during their planktonic life stage (Arzul *et al.,* 2010).

A lag time of at least 3 months is generally observed before detecting the parasite in disease free batches moved into infected areas.

2.3.5. Environmental factors

Survival of parasites purified and maintained in sea water is lower at 25°C than at 4°C or 15°C (Arzul *et al.,* 2009). High salinities (35, 40 and 45 ppt) appear to favour parasite survival (Arzul *et al.,* 2009). Prevalence shows an annual pattern that may differ according to areas. Prevalence of infection increases from autumn and shows a peak in late winter/early spring. Two peaks generally occurring in winter/spring and in autumn have been reported (Arzul *et al.,* 2006; Culloty & Mulcahy, 1996; Mérou *et al.,* 2023). Lower summer temperatures and higher summer salinities induce higher prevalence the following winter (Arzul *et al.,* 2006). *Ostrea edulis* appears to be more susceptible to *B. ostreae* following a period of lower food availability and lower salinities (Engelsma *et al.,* 2010).

2.3.6. Geographical distribution

Infection with *B. ostreae* has been found in Europe, North America (Carnegie & Cochennec-Laureau, 2004) and Oceania (Lane *et al.,* 2016).

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

Selective breeding has been shown to be effective in reducing susceptibility and mortality caused by *B. ostreae* (Lynch *et al.,* 2014; Naciri-Graven *et al.,* 1998).

2.4.5. Inactivation methods

Peracetic acid bath (0.001% and 0.005%) has been shown to reduce contamination of oysters by *B. ostreae* (Grizel, 1985). Bench scale experiment showed that a 94 mJ/cm2 UV C exposure inactivates up to 40% of *B. ostreae* isolated from infected oysters (Fernandez-Boo *et al.,* 2021).

2.4.6. Disinfection of eggs and larvae

No data available.

2.4.7. General husbandry

Mortalities caused by bonamiosis can be reduced using suspension culture, lower stocking densities or by culturing *Ostrea edulis* with *Magallana* (*Crassostrea) gigas,* which are not naturally susceptible to infection (Carnegie & Cochennec-Laureau, 2004). Oyster seed from hatcheries are preferred to seed from natural settlements which appears to have higher levels of parasites (Conchas *et al*., 2003). In an infected zone, harvesting larger oysters should allow reducing the parasite load in the population.

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) should be sampled by priority, to increase the chances of detecting infected oysters. For histology, only live (including moribund) oysters should be sampled.

Sampling of bivalves should be carried out 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 in late winter-early spring (Arzul *et al.,* 2006; Culloty & Mulcahy, 1996; Engelsma *et al.,* 2010).

3.2. Selection of organs or tissues

A 3–5 µm thick section of tissues including gills, mantle, gonad, and digestive gland, is used for diagnosis of *B. ostreae* by histology. Gills or heart are preferred for some tests, including imprints and PCR.

3.3. Samples or tissues not suitable for pathogen detection

Tissues other than gills, heart and mantle are less suitable.

3.4. Non-lethal sampling

No difference was observed between results obtained using real-time PCR from a mix of gill, mantle and digestive gland tissues and using real-time PCR from a biopsy of gills collected on anaesthetised oysters (Kamermans *et al.,* 2023).

Environmental DNA- and RNA-based approaches have been successfully developed allowing the detection of parasite DNA or RNA in sea water (Mérou *et al.,* 2020; von Gersdorff Jorgensen *et al.,* 2020). Although these methods allow detection in experimental conditions, their application in the field has not been validated (Mérou *et al.,* 2023).

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

Not applicable.

3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–100% (v/v) analytical-grade ethanol.

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. The effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, therefore 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. However, the detection of *B. ostreae* DNA was found similar between individuals and pools of five individuals when using a real-time PCR assay targeting the multiple copy 18S gene (Lane *et al.,* 2017).

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

LV = level of validation, refers to the stage of validation in the WOAH Pathway (chapter 1.1.2), Figures in brackets mean that partial data are available; NA = not available; PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively;
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. Imprints

Samples to be taken consist of heart (preferably the ventricle) or gills from fresh, gaping or freshly dead bivalves if they are sufficiently large. If bivalves are too small (as spat), imprints should be done using the entire individual.

After 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.

Imprints are generally less sensitive than PCRmethods (see Sections 6.1. and 6.2).

Infection with *Bonamia* *ostreae* is indicated by the presence of small spherical or ovoid organisms (2–5 µm wide) within haemocytes. However, the parasite might also occur extracellularly. These organisms show a basophilic cytoplasm often containing a lipid vacuole and an eosinophilic nucleus which is rather eccentric in the case of *B. ostreae* and rather centred in the case of *B. exitiosa* (colours of cytoplasm and nucleus may vary with the stain used). Parasitic cells can appear wider on imprints than on histological examination. Multinucleated cells can be observed (Balouet *et al.,* 1983; Bucke, 1988). The technique is not parasite species specific.

A positive result is indicative of infection with a *Bonamia* species.

4.2. Histopathology

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

Sections of tissue that include gills, digestive gland, mantle, and gonad should be fixed for 24 hours minimum 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.

Histology is generally less sensitive than PCRmethods (see Sections 6.1. and 6.2).

Infection with *Bonamia* *ostreae* is indicated by the presence of small cells of 2–5 µm wide within the haemocytes or free in the connective tissue or sinuses of gills, gut, digestive gland, gonad and mantle, often associated with an intense inflammatory reaction. Parasite cells could be observed in some epithelia including stomach or mantle epithelia (Balouet *et al.,* 1983). To avoid any doubt, the parasite has to be observed inside the haemocyte for a positive diagnosis. Although *B. ostreae* is slightly smaller than *B. exitiosa* and usually presents an eccentric nucleus, both species are difficult to distinguish in histology. No spore has been described in *B. ostreae* unlike *B. perspora* which has also a central to slightly eccentric nucleus. The technique is not species specific.

A positive result is indicative of infection with a *Bonamia* species.

4.3. Transmission electron microscopy

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

A small sized piece of tissue (1–2 mm) should be fixed in an appropriate fixative for at least 1 hour and then processed as described in section 5.4 of Chapter 2.4.0 *General information* (diseases of molluscs).

Infection with *B. ostreae* is indicated by the presence of parasites within the haemocytes. Different stages, including uninucleated, and rarely binucleated and plasmodial stages have been reported (Montes *et al*., 1994; Pichot *et al*., 1979). Intracellular structures include mitochondria, haplosporosomes, Golgi apparatus and persistent intranuclear microtubules. Two forms were described including a dense form rich in ribosomes and haplosporosomes and a light form, slightly larger with less dense cytoplasm and a nucleus showing a large nucleolus (Bucke, 1988; Pichot *et al.,* 1979).

Uninucleated *B. ostreae* stages are smaller than *B. exitiosa* or *B. perspora* ones and have larger haplosporosomes than other *Bonamia* spp (Hine *et al.,* 2014). They are also denser and have fewer lipoid bodies than other *Bonamia* species (Hine *et al.,* 2001).

4.4. Nucleic acid amplification

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). 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 sample can be diluted prior to PCR analyses (a 1/10 dilution allows to resolve most cases of PCR inhibition). Each sample should be tested in duplicate.

*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.

4.4.1. Real-time PCR

Three TaqMan PCR assays are available for the detection of *Bonamia* spp.: one targeting the ITS1 (internal transcribed spacer) (Corbeil *et al.,* 2006) and two targeting the 18S (small subunit rDNA) (Canier *et al.,* 2020; Marty *et al.,* 2006). The PCR assay developed by Canier *et al.* (2020) targets the 18S (small subunit rDNA) and allows the concomitant detection of *Bonamia* sp. and *Marteilia refringens* parasites.

Two other real-time PCR protocols have been developed to specifically detect *B. ostreae*: oneSYBR green PCR assay targeting the 18S-ITS1 region (Ramilo *et al.,* 2013), and a Taqman PCR assay targeting the actin gene (<https://www.eurl-mollusc.eu/SOPs>). These PCR assays allow the concomitant detection of *B. ostreae* and *B. exitiosa* parasites.

PCR assays are generally more sensitive than histology or cytology for the diagnosis of *B. ostreae* (see Sections 6.1. and 6.2). Real-time PCR usually have a better sensitivity than conventional PCR (see Sections 6.1. and 6.2).

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

|  |  |  |  |
| --- | --- | --- | --- |
| Pathogen/target gene | Primer/probe (5’–3’) | Concentration | Cycling parameters(a) |
| Method 1: Corbeil *et al.,* 2006; GenBank Accession No.: JN040831 |
| Taqman® PCR*Bonamia* spp./ITS-1 | ITS-For: CCC-TGC-CCT-TTG-TAC-ACA-CITS-Rev: TCA-CAA-AGC-TTC-TAA-GAA-CGC-GProbe Bon ITS: TTA-GGT-GGA-TAA-GAG-CCG-C (FAM MGB-NFQ) | 900 nM900 nM250 nM | 35 cycles of: 95°C/15 sec and 63.6°C/60 sec |
| Method 2: Marty *et al.,* 2006; GenBank Accession No.: AF192759 |
| Taqman® PCR*Bonamia* spp./18S | Fwd: CCC-GGC-TTC-TTA-GAG-GGA-CTARev: ACC-TGT-TAT-TGC-CCC-AAT-CTT-CProbe: CTG-TGT-CTC-CAG-CAG-A (FAM MGB-NFQ) | 800 nM800 nM250 nM | 40 cycles of: 95°C/15 sec and 60°C/60 sec |
| Method 3: Canier *et al.,* 2020; GenBank Accession No. AF192759 |
| Taqman® PCR*Bonamia* spp./18S | Bosp2-18S-F: CAG-GAT-GCC-CTT-AGA-TGC-TCBosp2-18S-R: GTA-CAA-AGG-GCA-GGG-ACG-TAProbe Bosp-18S-IN: TTG-ACC-CGG-CTT-GAC-AAG-GC (HEX-BHQ1) | 300 nM500 nM300 nM | 40 cycles of: 95°C/15 sec and 60°C/60 sec |
| Method 4: Ramilo *et al.,* 2013; GenBank Accession No. AF262995 |
| SYBR Green PCR*B. ostreae*/18S-ITS | BOSTRE-F: TTA-CGT-CCC-TGC-CCT-TTG-TABOSTRE-R: TCG-CGG-TTG-AAT-TTT-ATC-GT | 300 nM300 nM | 35 cycles of: 95°C/30 sec and 55°C/45 sec, 72°C/60 secMelt curve from 55°C to 95°C with 0.5°C increment/sec |
| Method 5: EURL for mollusc diseases; GenBank Accession No: AF192759 |
| Taqman® PCR*B. ostreae/*actin | BO2\_F: AAA-TGG-CCT-CTT-CCC-AAT-CTBO2\_R: CCG-ATC-AAA-CTA-GGC-TGG-AABO2 probe: TGA-CGA-TCG-GGA-ATG-AAC-GC (HEX BHQ1) | 300 nM300 nM200 nM | 40 cycles of: 95°C/15 sec and 60°C/20 sec |

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

4.4.2. Conventional PCR

Three conventional PCR protocols targeting the 18S (small subunit rDNA) have been developed for the detection of *Bonamia* sp.(Carnegie *et al.,* 2000; Cochennec *et al*., 2000) or *B. ostreae* (Engelsma *et al.,* 2010).

The PCR assay described by Carnegie *et al.* (2000) amplifies most of the identified *Bonamia* spp. including *B. ostreae* and *B. exitiosa*, while the Cochennec *et al.* (2000) PCR amplifies *Bonamia* spp. and several members of *Haplosporidia.* The Engelsma *et al.* (2010) PCR was shown not to detect *B. exitiosa* and *Haplosporidium armoricanum.*

Conventional PCR assays were generally more sensitive than histology or cytology (see Sections 6.1. and 6.2) although Lynch *et al.* (2008) found that heart imprint was more sensitive than the conventional PCR from Cochennec *et al.* (2000). Under certain circumstances, the primers from Cochennec *et al.* (2000) can generate a 295 bp non-specific product (Engelsma *et al.,* 2014).

***Primer sequences***

|  |  |  |  |
| --- | --- | --- | --- |
| Pathogen /target gene | Primer (5’–3’) | Concentration | Cycling parameters(a) |
| Method 1: Carnegie *et al.,* 2000 modified in Carnegie *et al.,* 2008; amplicon size [760 bp] |
| *Bonamia* spp./18Samplifies most of the identified *Bonamia* spp including *B. ostreae* and *B. exitiosa* | CF: CGG-GGG-CAT-AAT-TCA-GGA-ACCR: CCA-TCT-GC-TGGA-GAC-ACA-G | 250 nM250 nM | 35 cycles of: 95°C/1min and 59°C/1 min and 72°/1min |
| Method 2: Cochennec *et al.,* 2000; amplicon size [300 bp] |
| *Bonamia* spp./18Samplifies all identified *Bonamia* spp. and several members of *Haplosporidia* | BO: CAT-TTA-ATT-GGT-CGG-GCC-GCBOAS: CTG-ATC-GTC-TTC-GAT-CCC-CC  | 1 µM1 µM | 30 cycles of: 95°C/60 sec, 55°C/60 sec, 72°C/60 sec |
| Method 3: Engelsma *et al.,* 2010; amplicon size [352 bp] |
| *B. ostreae*/18S | BoosF03: CAA-TGG-TGC-GTT-CAA-CGA-GTBoosR03: GGG-TTC-GCG-GTT-GAATTT-TA | 400 nM400 nM | 40 cycles of: 95°C/30 sec, 58°C/30 sec, 72°C, 45 sec |

(a)A denaturation step at 94-95°C prior to cycling and a final elongation step at 72°C
(between 5 and 10 minutes) must be included.

The PCR methods 1 and 2 are not specific for *B. ostreae*. Sequence analysis of the amplicons must be used to confirm identity. Amplicons obtained by method 2 can be digested with the Bgl enzyme which allows to distinguish two profiles: *B. ostreae* (two bands of 120 and 180 bp) and *B. exitiosa* (not digested).

4.4.3. Other nucleic acid amplification methods

None available.

4.5. Amplicon sequencing

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

Sequencing is recommended as one of the final steps for confirmatory diagnosis. Targeted regions are 18S, ITS1 and actin. Although the sequences are available in the public gene banks, it is recommended to refer such cases to the appropriate WOAH Reference Laboratory.

4.6. *In-situ* hybridisation

Samples to be taken: live or freshly dead oysters.

Several *in situ* hybridisation (ISH) protocols targeting the 18S have been developed.

The first one (Cochennec *et al*., 2000) allows a detection at the *Bonamia* genus level and uses a 300 bp labelled probe produced by PCR.

Two ISH protocols were designed to specifically detect *B. ostreae* (Carnegie *et al.,* 2003; Hill *et al.,* 2014), and rely on labelled oligonucleotide probes. However, the ISH protocol from Carnegie *et al.* (2003) should also detect *B. exitiosa* according to probe sequence analysis.

|  |  |  |  |
| --- | --- | --- | --- |
| Reference | Pathogen/target | ISH probe type | ISH probe  |
| Method 1Cochennec *et al.,* 2000 | *Bonamia* spp. and several members of *Haplosporidia*18S | Labelled BO–BOAS amplicons | BO–BOAS PCR product (300bp) |
| Method 2:Carnegie *et al.,* 2003 | *B. ostreae*, and *B. exitiosa*18S | Labelled oligonucleotides | UME-BO-1: CGA-GGC-AGG-GTT-TGT; UME-BO-2: GGG-TCA-AAC-TCG-TTG-AACUME-BO-3: CGC-TCT-TAT-CCA-CCT-AAT |
| Method 3Hill *et al.,* 2014 | *B. ostreae*18S | Labelled oligonucleotides | Bost171: CCG-CCG-AGG-CAG-GGT-TTG-T |

*Technical procedure*

The first steps in the technical procedure follow the recommendations described in chapter 2.4.0.

Subsequent steps concerning Method 1 (Cochennec *et al.,* 2000) are that the probe is produced by PCR using the previously described primer pair Bo–Boas (Section 4.4.2) with digoxigenin incorporation and the PCR is performed as described in the section on PCR except that DIG dUTP 25 mM is added to the reaction mixture. The detection steps are performed according to the manufacturer’s instructions. In other protocols, probes consist in digoxigenin-labelled nucleotides.

Slides are dehydrated by immersion in an ethanol series and air dried. The slides are then covered with hybridisation buffer (4 × SSC [standard saline citrate; 60 mM NaCl, 600 mM NaCl, pH 7], 50% formamide, 1 × Denhardt’s solution, 250 µg ml–1 yeast tRNA, 10% dextran sulphate) containing approx. 20 ng of the digoxigenin-labelled probe (1–2 µl of the probe produced by PCR, or 1 µl at 100 µm of labelled nucleotides). Sections are covered with *in-situ* plastic cover-slips and placed for 5 minutes at 95°C. Slides are then cooled on ice for 1– 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 an appropriate staining, rinsed in tap water, immersed in 95% and 100% ethanol for 30 seconds for each, rinsed for 10–30 seconds in xylene and cover-slips are applied using an appropriate mounting medium.

In Carnegie et al. (2003)after proteinase K treatment, slides are washed in several baths including PBS plus 0.2% glycine for 5 minutes, acetylated using 5% anhydrous acetic in 0.1 M triethanolamine/HCl (pH 8), for 10 minutes at room temperature, washed again in PBS for 10 minutes and lastly equilibrated in 5 × SET (750 mM NaCl, 6.4 mM EDTA, 100 mM Tris Base) for 10 minutes at room temperature. Slides are then covered with 200 µl of prehybridisation buffer (5 × SET, 0.02% bovine serum albumin, 0.025% sodium dodecyl sulphate [SDS]) for 30 minutes at 45°C. Prehybridisation buffer is replaced with 10 to 12 µl of the prehybridisation buffer containing 2–10 ng µl–1 of the oligonucleotides and slides are incubated overnight in a humid chamber at 45°C. Slides are then washed three times in 0.2 × SET for 5 minutes at 42°C, air dried and mounted before being examined using an epifluorescence microscope at ×600–1000.

*Interpretation of results:*

A positive result corresponds to labelled parasites inside the haemocytes, with all negative controls (including non-infected sample and no probe ISH reaction control) negative and all positive controls (including infected sample) positive. In addition, non-specific probe such as SSUrDNA can be used to verify the integrity of DNA in paraffin blocks.

4.7. Immunohistochemistry

Not available.

4.8. Bioassay

Not available.

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

Although an immunofluorescent technique based on monoclonal antibodies was developed It has never been validated and it is no longer available (Carnegie & Cochennec-Laureau, 2004).

4.10. Other methods: agent purification

*Bonamia ostreae* can be purified from highly infected oysters (Mialhe *et al.,* 1988). All organs are homogenised except the adductor muscle, and parasites are concentrated by differential centrifugation on sucrose gradients and then purified by isopycnic centrifugation on a Percoll gradient.

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 *B. ostreae*. Histology, tissue imprint and conventional PCR can also be used (see Table 4.1)

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-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 *Bonamia ostreae* shall be suspected if at least one of the following criteria is met:

i) Observation of parasite cells in tissue imprints

ii) Observation of parasite cells in tissue sections with or without histopathology characteristic of the pathogen

iii) Positive result by conventional PCR

iv) Positive result by real-time PCR

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *Bonamia ostreae* is considered to be confirmed if the following criterion is met:

i) Positive result by tissue imprints or histology followed by real-time PCR or by conventional PCR and sequencing or by species-specific *in-situ* hybridisation

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 *Bonamia ostreae* shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with the disease as described in this chapter

ii) Observation of parasite cells in tissue imprints

iii) Observation of parasite cells in tissue sections with or without histopathology characteristic of the pathogen

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 *Bonamia ostreae* is considered to be confirmed if the following criterion is met:

i) Positive result by real-time PCR or by conventional PCR and sequencing or by species-specific *in-situ* hybridisation

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *B. ostreae* 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 *B. ostreae*, 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.

Data on analytical performances (stage 1 validation) are often missing for diagnostic tests described in this chapter: the limit of detection is rarely available, and the inclusivity of molecular assays is not fully evaluated (missing information on the detection of *Bonamia* sp. lineages/species other than *B. ostreae* and *B. exitiosa*).

Diagnostic sensitivity (DSe) and specificity (DSp) (stage 2 validation) are available for most diagnostic tests. However, these values depend on the studied mollusc population (host species, prevalence, intensity of infection, etc.), the protocol (tissue analysed, DNA extraction, use of cut-off value for PCR assays, etc.) and test purpose. Additionally, as no gold standard exists for the detection of *B. ostreae*, several approaches can be used for DSe and DSp estimation, such as the use of a combination of tests to establish reference results or latent class analysis (maximum likelihood or Bayesian method). If Bayesian latent class is used, the analysis can incorporate prior knowledge about the performance of compared diagnostic tests. The choice of the overall approach used will have an impact on DSe & DSp estimates. It is therefore complex to compare DSe/DSp estimates from different studies.

Few assays were evaluated for their reproducibility (stage 3 validation). Two real-time PCR (Canier *et al.,* 2020, and EURL, 2023) were evaluated in the context of interlaboratory comparison tests. Additionally, a study comparing conventional PCR, ISH, heart imprint and histology in three laboratories showed that conventional PCR produces the highest rate of positive *Bonamia ostreae* detection but also had the lowest agreement amongst laboratories (Flannery *et al.,* 2014).

6.3.1. For presumptive diagnosis of clinically affected animals

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 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

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Histology | Surveillance | Flat oysters from three farms in western Canada (spats sourced from Washington, USA, where *B. ostreae* is endemic). Low prevalence populations | Tissue section | *Ostrea edulis*(1–2.5 years) | 56%(607) | 100%(607) | Combination histology and real-time PCR (DSe: 56%, DSp: 100%) | Marty *et al.,* 2006 |
| Surveillance | Flat oysters produced in hatchery derived from five origins, deployed in the field, in a *B. ostreae & B. exitiosa* endemic area (Galicia, Spain). High prevalence populations | Tissue section | *Ostrea edulis*(2–3 years) | 64%(137) | 98%(137) | Real-time (DSe 99%, DSp 72%) and conventional PCRMaximum likelihood latent class analysis (TAGS) | Ramilo *et al.,* 2013 |
| Surveillance  | Flat oysters from three farms in South Australia (high prevalence populations 60–90%, but low intensity of infection) | Tissue section | *Ostrea angasi* | 76%(400) | 93%(400) | Real-time PCR Corbeil *et al.,* 2006 (DSe: 69%, DSp: 93%) and Heart imprint (DSe 61%, DSp 60%) Bayesian latent class analysis | Buss *et al.,* 2019 |
| Tissue imprints | Surveillance  | Flat oysters from 3 farms in South Australia (high prevalence populations 60–90%, but low intensity of infection) | Heart | *Ostrea angasi* | 61%(400) | 60%(400) | Histology (DSe: 76%, DSp: 93%) and real-time PCR Corbeil *et al.,* 2006 (DSe: 69%, DSp: 93%). Bayesian latent class analysis | Buss *et al.,* 2019 |
| Conventional PCR *Bonamia* sp.(Cochennec *et al.,* 2020) | Surveillance | Eight batches of 30 flat oysters, Spain (tested by two laboratories) (total prevalence 10–30%) | NA | *Ostrea edulis* | 93%(240) | 85–90%(240) | Combination histology and gill imprints (DSe: 64-69%, DSp: 97.5%) | Balseiro *et al.,* 2006 |
| Surveillance | Flat oysters from the 3 main production sites in France representative of three different levels of *B. ostreae* prevalence (very low, low, high) | Gills and digestive gland tissues | *Ostrea edulis*(1–3 years) | 82.8%(349) | 98.7%(349) | Real-time PCR (DSe 77.5%, DSp 98.4%). Bayesian latent class analysis | Canier *et al.,* 2020 |
| Taqman real-time PCR *Bonamia* sp. | Surveillance | Flat oysters from three farms in western Canada (spats sourced from Washington, USA, where *B. ostreae* is endemic). Low prevalence populations | Heart | *Ostrea edulis*(1-2.5 years) | 88%(607) | 99%(607) | Combination histology and real-time PCR. Histology (DSe: 56%, DSp: 100%) | Marty *et al.,* 2006 |
| Taqman real-time PCR *Bonamia* sp.(Corbeil *et al.,* 2006) | Surveillance  | Flat oysters from three farms in South Australia (high prevalence populations 60–90%, but low intensity of infection) | Mantle, gill, heart(DNA tested pure and 1/10 diluted) | *Ostrea angasi* | 69%(400) | 93%(400) | Histology (DSe: 76%, DSp: 93%) and heart smear (DSe: 61%, DSp: 60%). Bayesian latent class analysis | Buss *et al.,* 2019 |
| Taqman real-time PCR*Bonamia* sp. | Surveillance | Flat oysters from the three main production sites in France representative of three different levels of *B. ostreae* prevalence (very low, low, high) | Gills and digestive gland tissues | *Ostrea edulis*(1–3 years) | 77.5%(349) | 98.4%(349) | Conventional PCR (DSe: 82.8%, DSp: 98.7%). Bayesian latent class analysis | Canier *et al.,* 2020 |
| SYBR Green real-time PCR *B. ostreae* | Surveillance | Flat oysters produced in hatchery derived from five origins, deployed in the field, in a *B. ostreae & B. exitiosa* endemic area (Galicia, Spain). High prevalence populations | Gills tissues | *Ostrea edulis*(2–3 years) | 99%(137) | 72%(137) | Histology (DSe: 64%, DSp: 98%) and conventional PCR). Maximum likelihood latent class (TAGS)  | Ramilo *et al.,* 2013 |

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of animals used in the validation study,
PCR: = polymerase chain reaction.
(TAGS programme, Pouillot *et al.,* 2002)

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\* \*

**NB:** There is a WOAH Reference Laboratory for infection with *Bonamia ostreae*
(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 *Bonamia ostreae*

**NB:** First adopted in 1995 as bonamiosis. Most recent updates adopted in 2021 (Sections 2.2.1 and 2.2.2).

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