Annex 24. Item 9.3.1. – Chapter 2.4.2. ‘Infection with *Bonamia exitiosa*’

Chapter 2.4.2.

infection with *Bonamia exitiosa*

1. Scope

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

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

*Bonamia exitiosa* is a haplosporidian protozoan parasite (Arzul & Carnegie, 2015; Carnegie & Cochennec-Laureau, 2004) infecting haemocytes of several oyster species, causing disease and mortality (Cranfield *et al.,* 2005; Dinamani *et al.,* 1987).~~Since the original description of the parasite in New Zealand in the mid-1980s,~~ *~~B. exitiosa~~* ~~and~~ *~~B. exitiosa~~*~~-like microcells have been described in various locations globally. Species assignment was based primarily on the sequence of the ITS rDNA locus of the ribosomal DNA gene complex, as the available data on histology ultrastructure and molecular sequences was insufficient to discriminate unequivocally between species (Hill et al. 2010b).~~

2.1.2. Survival and stability in processed or stored samples

No data available

2.1.3. Survival and stability outside the host

No data available.

2.2. Host factors

2.2.1. Susceptible host species

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

| **Family** | **Scientific name** | **Common name** |
| --- | --- | --- |
| Ostreidae | *Crassostrea virginica* | eastern oyster |
|  | *Magallana* (syn. *Crassostrea*) *ariakensis* | Ariake cupped oyster |
|  | *Ostrea angasi* | Australian mud oyster |
|  | *Ostrea chilensis* | Chilean flat oyster |
|  | *Ostrea edulis* | European flat oyster |
|  | *Ostrea equestris* | crested oyster |
|  | *Ostrea lurida* | Olympia oyster |
|  | *Ostrea puelchana* | Argentinean 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. exitiosa* according to Chapter 1.5 of the *Aquatic Code* are: ~~dwarf oyster~~~~(~~*~~Ostrea stentina~~*~~).~~

| **Family** | **Scientific name** | **Common name** |
| --- | --- | --- |
| Ostreidae | *Ostrea stentina* | dwarf 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** |
| --- | --- | --- |
| Ostreidae | *Magallana* (syn. *Crassostrea*) *gigas* | Pacific cupped oyster |
|  | *Saccostrea glomerata* | Sydney rock oyster |

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

Juveniles and adults are susceptible to infection however, prevalence and infection intensity are generally higher in individuals of 2 years of age. In *O. edulis*, *B. exitiosa* DNA has also been detected in larvae (Arzul *et al.,* ~~2010~~ 2011; Helmer *et al.,* 2020). *Bonamia exitiosa* is particularly pathogenic in young *M. ariakensis*, <50 mm in shell height (Bishop *et al.,* 2006).

2.2.4. Distribution of the pathogen in the host

*Bonamia exitiosa* is an intrahaemocytic protozoan, but it can be observed extracellularly (Dinamani *et al.,* 1987). Infection is systemic with the protozoan found in several organs and especially in the connective tissues of gills and mantle (Hine, 1991a). In *O. angasi*, the parasite has been observed in the gills, mantle and gonad and particularly in the connective tissue of the digestive gland (Buss *et al.,* 2020a). In *O. edulis*, the parasite is associated with heavy haemocytic infiltration and appears in the connective tissue of various organs mostly within haemocytes, but sometimes outside host cells (Abollo *et al.,* 2008). In *O. stentina*, haemocytosis was not observed in animals found to be infected with the parasite (Hill *et al.,* 2010).

2.2.5. Aquatic animal reservoirs of infection

Susceptible species(see Section 2.2.1) should be considered potential reservoirs.

2.2.6. Vectors

None known.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Mortality in *O. chilensis* occurs concurrently with the highest infection intensity, particularly in association with high intensity apicomplexan infections (Hine, 2002; Hine & Wesney, 1994). The disease seems to kill more than 80% of the oysters as the wave of infection passes through an oyster bed over a period of 2–3 years (Cranfield *et al.,* 2005). In *O. angasi*, >85% mortality was observed in oysters after 40 days of exposure with infected oysters (Buss *et al.,* 2020a).

Prevalence is variable in *O. chilensis* (from 0% to nearly 80%) (Cranfield *et al.,* 2005). In the Southern Hemisphere, infection with *B. exitiosa* shows the highest prevalence from January to April, with the parasite barely detectable in September and October (Hine, 1991a). Stressors such as exposure to extreme temperatures (below 7°C or above 26°C), high salinity (40 ppt), starvation (prolonged holding in filtered sea water), handling (vigorous stirring four times per day), or heavy infection with an [apicomplexan](http://www.pac.dfo-mpo.gc.ca/sci/shelldis/pages/apicomplexnzoy_e.htm) (Hine, 2002), can affect the disease dynamics of *B. exitiosa* in *O. chilensis* (Hine *et al.,* 2002).

Prevalence is variable in *O. edulis* in which co-infection with *B. ostreae* was reported (Abollo *et al.,* 2008). In Galicia (Spain), the maximum reported prevalence of *B. exitiosa* in *O. edulis* was 34% in one batch collected in October (Abollo *et al.,* 2008). Despite some prevalence differences observed between sampling dates, it is not presently possible to determine the annual infection pattern of flat oysters with *B. exitiosa* in Europe.

In *Ostrea angasi*, no clear seasonal pattern was described and prevalence increased over time from 8 to 40% after 3 months to 57 to 88% after 1 year, depending on farming site (Buss *et al.,* 2020c).

2.3.2. Clinical signs, including behavioural changes

Clinical signs include dead or gaping oysters.

2.3.3 Gross pathology

Most live infected oysters appear normal, but sometimes the gills can appear to be eroded (Dinamani *et al*., 1987).

2.3.4. Modes of transmission and life cycle

Transmission by infective stages carried passively on water currents between oyster beds is suspected (Cranfield *et al.,* 2005; Hine, 1996). Studies with *O. chilensis* have shown that transmission of the parasite directly from host to host is possible; Hine (1991a; 1991b) has shown that released infective particles are ingested by oysters and enter the haemolymph from the gut. Infective particles are phagocytosed by agranular haemocytes, and are able to resist lysis within the haemocyte (Hine & Wesney, 1994).

Parasite DNA has been detected in larvae incubated in the palleal 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 (Helmer *et al.,* 2020).

2.3.5. Environmental factors

Experimental studies using variations in temperature and salinity as stressors (Hine *et al.,* 2002) showed that prevalence was higher in oysters kept for a short period (14 days) in warm water (25–26°C for 1 hour daily) or in hypersaline (39–40 ppt) water compared with cold water (7°C for 1 hour daily) and to hyposaline water (15 ppt).

In *O. chilensis*, prevalence shows an annual pattern with two peaks reported in April (early autumn) and August (winter) (Hine, 1991a). The evolution of *B. exitiosa* in *O. edulis* or *O. stentina* according to the season has not been studied.

Increased water temperature increases the risk of death of *O. angasi* due to *B. exitiosa* infection particularly when it is combined with other stressors – both starvation and increased motion (Bradley *et al.,* 2020).

2.3.6. Geographical distribution

Infection with *B. exitiosa* has been reported ~~from~~ in *O. chilensis* in Oceania (Dinamani *et al*., 1987; Doonan *et al.,* 1994); in *O. angasi* in Oceania (Corbeil *et al.,* 2006b; Hine, 1996; Hine & Jones, 1994); in *O. edulis* in Europe (Abollo *et al.,* 2008; Narcisi *et al.,* 2010); and in *O. stentina* in Africa (Hill *et al.,* 2010).

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

40,000 ppm chlorine for 10 minutes and 2000 ppm iodine for 1 minute inactivate 100% of *B. exitiosa* isolated from infected oysters (Buss *et al.,* 2020b).

2.4.6. Disinfection of eggs and larvae

No data available.

2.4.7. General husbandry

Development of lighter dredges and less damaging fishing strategies should reduce the chance of disease outbreaks by lowering disturbance (Cranfield *et al.,* 2005). Avoiding stressors such as exposure to extreme temperatures (below 7 or above 26°C) and high salinity (40 ppt), starvation, handling, or heavy infection with other parasites, as well as decreasing stocking density, should mitigate the impact of the disease (Cranfield *et al.,* 2005; Hine *et al.,* 2002).

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

Sampling should be carried out when prevalence is known to be at a maximum, or during periods of higher water temperature e.g. between January and April in the Southern Hemisphere (Hine, 1991a).

3.2. Selection of organs or tissues

A 3–5 µm thick section of tissue that includes a sample of gills, mantle, gonad~~,~~ and digestive gland, is used for histological examination. Gills or heart are preferred for some tests, including ~~such as~~ imprints and PCR. ~~For PCR in~~ *~~O. edulis~~* ~~it is recommended to include gills and gonad.~~ More than 1 organ (including gills, heart and gonad) should be used for PCR-based diagnosis of *B. exitiosa*, to maximise the sensitivity (Ramilo *et al.,* 2014).

3.3. Samples or tissues not suitable for pathogen detection

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

3.4. Non-lethal sampling

None.

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 80% (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.

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 |  | +++ | +++ | 2 |  |  |  |  |
| Transmission electron microscopy |  |  |  |  |  |  |  |  | + | + | + | NA |
| Real-time PCR | +++ | +++ | +++ | 3 | +++ | +++ | +++ | 2 | +++ | +++ | +++ | NA |
| Conventional PCR | ++ | ++ | ++ | 2 | +++ | +++ | +++ | NA |  |  |  |  |
| Conventional PCR followed by amplicon sequencing |  |  |  |  |  |  |  |  | +++ | +++ | +++ | NA |
| *In-situ* hybridisation  |  |  |  |  | + | + | + | 1 |  |  |  |  |
| 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 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.
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 (such 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 (in methanol or 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.

Infection with *B. exitiosa* isindicated by the presence of small spherical or ovoid organisms (2–5 µm wide) within the 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 central unlike the *B. ostreae* nucleuswhichis rather eccentric (colours of cytoplasm and nucleus may vary with the stain used). Parasitic cells can appear larger on imprints than on histological examination. Multinucleated cells can be observed (Abollo *et al.,* 2008; Hine *et al.,* 2001). The technique is not parasite species specific.

A positive result is indicative of infection with a *Bonamia* sp~~ecies~~.

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.

Infection with *B. exitiosa* is indicated by the presence of parasites as small cells (2–5 µm in diameter) within the haemocytes or free in the connective tissue or sinuses of the gills, gonads, digestive gland, gut and mantle. The parasite causes different lesions according to it host. It is often associated with an intense disseminated haemocyte infiltration in *O. chilensis* but intense focal haemocyte infiltration in *O. angasi* in which it is epitheliotrophic (Engelsma & Hine, 2009). In *O. edulis*, it is associated with haemocyte infiltration of the connective tissues surrounding the digestive gland and the mantle (Longshaw et al., 2013). To avoid any doubt, the parasite has to be observed inside the haemocyte for a positive diagnosis.

*B. exitiosa* is generally larger than *B. ostreae* and often has a central or subcentral nucleus (Lane *et al.,* 2016). Plasmodia stages characterised by irregular shape were noted in the haemocyte cytoplasm but, unlike *B. perspora,* no spore has been described in *B. exitiosa*. The technique is not species-specific.

Positive result is indicative of infection with a *Bonamia* sp~~ecies~~.

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. exitiosa* is indicated by the presence of parasites within the haemocytes. Different stages, including uninucleated, binucleated and plasmodial stages have been reported; moreover, B. exitiosa has a large amoeboid trophic stage, apparently not present in B. ostreae. Intracellular structures include mitochondria, haplosporosomes, Golgi apparatus and persistent intranuclear microtubules. In *O. chilensis*, four parasite developmental stages have been described in infected oysters corresponding to dense forms, intermediate forms, plasmodial forms and vacuolated forms (Hill *et al.,* 2010; Hine, 1991b; Hine *et al.,* 2001).

Uninucleated stages of *B.* *exitiosa* are slightly larger in size in comparison with *B.* *ostreae* and have more haplosporosomes, mitochondrial profiles and lipoid bodies per ultrastructure section, as well as smaller tubulo-vesicular mitochondria. However, this stage is smaller in comparison with *B. perspora* which has also smaller haplosporosomes (Hine *et al.,* 2001; 2014).

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 samples can be diluted prior to PCR analyses (a 1/10 dilution usually resolves 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) region (Corbeil *et al.,* 2006a) 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* spp. and *Marteilia refringens* parasites.

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

PCR assays are generally more sensitive than histology and/or cytology for the diagnosis of *B. exitiosa* (see Sections 6.1. and 6.2) although Buss *et al.* (2019) found that histology was more sensitive than real-time PCR in farmed populations of *Ostrea angasi*. Real-time PCR assays usually have higher sensitivity than conventional PCR assays (see Sections 6.1. and 6.2).

***Primers and probes* (*sequence*)**

Method 1: Corbeil *et al.,* 2006a; GenBank Accession No.: DQ312295

Method 2: Marty *et al.,* 2006; GenBank Accession No.: DQ312295

Method 3: Canier *et al.,* 2020; GenBank Accession No.: EU016528

Method 4: Ramilo *et al.,* 2013; GenBank Accession No: DQ312295

Method 5: EURL for mollusc diseases (2023); GenBank Accession No: KM073106

|  |  |  |  |
| --- | --- | --- | --- |
| Pathogen/target gene | Primer/probe (5’–3’) | Concentration | Cycling parameters(a) |
| TaqMan® PCR*Bonamia* spp./ITS-1Method 1 | ITS-For: CCC-TGC-CCT-TTG-TAC-ACA-CCITS-Rev: TCA-CAA-AGC-TTC-TAA-GAA-CGC-GProbe BonITS: 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 |
| TaqMan® PCR*Bonamia* spp./18SMethod 2 | Fwd: CCC-GGC-TTC-TTA-GAG-GGA-CTARev: ACC-TGT-TAT-TGC-CCC-AAT-CTT-C Probe: 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 |
| TaqMan® PCR*Bonamia* spp./18SMethod 3 | 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 nm.500 nM300 nM | 40 cycles of: 95°C/15 sec and 60°C/60 sec |
| SYBR Green PCR*B. exitiosa/*18S-ITSMethod 4 | BEXIT-F: GCG-CGT-TCT-TAG-AAG-CTT-TGBEXIT-R: AAG-ATT-GAT-GTC-GGC-ATG-TCT | 300 nM300 nM | 35 cycles of: 95°C/30 sec and 58°C/45 sec, 72°C/60 secMelt curve from 58°C to 95°C with 0.5°C increment/sec |
| TaqMan® PCR*B. exitiosa/*actinMethod 5 | BEa\_F: GAC-TTT-GAC-CAT-CGG-AAA-CG BEa\_R: ATC-GAG-TCG-TAC-GCG-AGT-CT BEa\_probe GGC-AGC-GAA-TCG-ATG-GGA-AT (FAM-BHQ-1) | 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

Two conventional PCR protocols targeting the 18S (small subunit rDNA) have been developed for the detection of *Bonamia* spp*.* (Carnegie *et al.,* 2000; Cochennec *et al*., 2000). Conventional PCRs are generally more sensitive than histology or cytology (see Sections 6.1. and 6.2). Under certain circumstances, the Cochennec *et al.* (2000) primers generate a 295 bp non-specific product of approximately the same size as the expected product of 300 bp (Engelsma *et al.,* 2014).

***Primer sequence*s**

Method 1: Carnegie *et al.,* 2000 modified in Carnegie *et al.,* 2008; amplicon size [760 bp]; GenBank Accession No.: AF262995

Method 2: Cochennec *et al.,* 2000; amplicon size [304 bp]: GenBank Accession No.: AF192759

Method 3: Ramilo *et al.,* 2013; amplicon size [246 bp], GenBank Accession No: DQ312295

|  |  |  |  |
| --- | --- | --- | --- |
| Pathogen/target gene | Primer (5’–3’) | Concentration | Cycling parameters(a) |
| *Bonamia* spp./18Samplifies most of the identified *Bonamia* spp. including *B. ostreae* and *B. exitiosa*Method 1 | CF: CGG-GGG-CAT-AAT-TCA-GGA-ACCR: CCA-TCT-GCT-GGA-GAC-ACA-G 250 nM 250 nM |  35 cycles of: 95°C/1 min and 59°C/1 min and 72°/1 min |
| *Bonamia* spp./18Samplifies all identified *Bonamia* spp. and several members of HaplosporidiaMethod 2 | 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 |
| *Bonamia exitiosa*/18S-ITS1Method 3 | BEXIT-F GCG-CG-TTC-TTA-GAA-GCT-TTGBEXIT-R AAG-ATT-GAT-GTC-GGC-ATG-TCT | 0.3 µM0.3 µM | 35 cycles of: 94°C/30sec, 58°C/45sec, 72°C/1 min  |

(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* I 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.

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 consist of live or freshly dead oysters.

Several *in-situ* hybridisation protocols have been developed, two targeting the 18S and one the ITS1.

The first one (Cochennec *et al*., 2000) allows 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. exitiosa* (Hill *et al.,* 2010; Ramilo *et al.,* 2014) but should also detect closely related parasites (belonging to the “clade *B. exitiosa*”). These assays rely on digoxigenin-labelled oligonucleotide probes.

| Reference | Pathogen/target | ISH probe type | ISH probe  |
| --- | --- | --- | --- |
| Method 1Cochennec *et al.,* 2000 | *Bonamia* spp. and several members of Haplosporidia18S | Labelled BO–BOAS amplicons | BO–BOAS PCR product (300bp) |
| Method 2:Hill *et al.,* 2010 | *Bonamia exitiosa* and closely related *Bonamia* spp. 18S | Three labelled oligonucletotides | CaBon166: CGA-GCA-GGG-TTT-GTC-ACG-TATCaBon461: TTC-CGA-ATA-GGC-AAC-CGA-AGCaBon1704: CAA-AGC-TTC-TAA-GAA-CGC-GCC  |
| Method 3:Ramilo *et al.,* 2014 | *Bonamia exitiosa* and closely related *Bonamia* spp. ITS1 | Labelled oligonucletotides | BEX\_ITS: CAA-AGA-TTG-ATG-TCG-GCA-TG |

*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~~ of 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 stain~~ing~~, 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.

*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.)

Not available.

4.10. Other methods

Not 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 *B. exitiosa*. 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 *B. exitiosa* 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 *B. exitiosa* is considered to be confirmed if at least one of the following criteria ~~criterion~~ is met:

1. ~~Positive result by tissue imprints or histology followed by real-time PCR or by conventional PCR and sequencing~~

i) Positive result by real-time PCR and conventional PCR and sequencing

ii) Positive result by real-time PCR and *in-situ* hybridisation

iii) Positive result by tissue imprints, histology or *in-situ* hybridisation, and positive result by 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 *B. exitiosa* 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

vi) Positive result by *in-situ* hybridisation

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *B. exitiosa* is considered to be confirmed if at least one of the following criteria ~~criterion~~ is met:

i) Positive result by real-time PCR ~~or~~ and by conventional PCR and sequencing

ii) Positive result by real-time PCR and *in-situ hybridisation*

iii) Positive result by tissue imprints, histology or *in-situ hybridisation*, and positive result by conventional PCR followed by sequence analysis

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *B. exitiosa* are provided in Tables 6.3.1. and 6.3.2. This information can be used for the design of surveys for infection with *B. exitiosa*, 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 always fully evaluated (missing information on the detection of *Bonamia* spp. 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. exitiosa*, 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.

Real-time PCR is generally considered as the most sensitive method except in some particular cases as for example for the diagnostic of *Bonamia* spp. in a population of farmed *O. angasi* in Australia, where histology was found to be more sensitive (Buss *et al.,* 2019). This population was characterised by a high *Bonamia* prevalence but low intensity of infection with focal lesions. The fact that PCR diagnosis is performed in a small part of tissue could explain this result.

Two real-time PCR (Canier *at al.,* 2020 and EURL, 2023) were evaluated for their reproducibility (stage 3 validation) in the context of interlaboratory comparison tests.

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 |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| TaqMan PCR *Bonamia* spp. (Corbeil *et al.,* 2006a) (with epidemiological Ct cut-off) | Diagnosis | Two farms in *B. exitiosa* endemic areas in Australia: a coastal lease with *B. exitiosa* associated mortalities, a land-based hatchery with no *B. exitiosa* associated mortalities (prevalences ~30 and 60%) | Gills | *Ostrea angasi* | 93.5% (232) | 92.2% (232) | Histology Bayesian latent class analysis  | Bradley *et al*., 2020 |
| Histology | Diagnosis | Tissue slide | 50.8% (232) | 98.2% (232) | Taqman PCR Bayesian latent class analysis |

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 | 28 flat oysters from one site in New Zealand (high prevalence 60–96%) | Tissue section  | *O. chilensis* | 44.4% (28) | 100% (28) | Combination conventional PCR and ISH (DSe & DSp: 100%) | Diggles *et al.,* 2003 |
| 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: 88%, DSp: 99%) | 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) | 54% (137) | 96% (137) | Real-time PCR (DSe: 100%, DSp: 77%) and conventional PCR. Maximum likelihood latent class analysis (TAGS) | Ramilo *et al.,* 2013 |
| Surveillance | 30 flat oysters from an area affected by *B. ostreae* and *B. exitiosa* in Galicia, Spain. High prevalence populations | Tissue section | *Ostrea edulis* | 63% (30) | 88% (30) | ISH (DSe: 82%, DSp:88%), PCR-RFLP (DSe 91%, DSp 100%), real-time PCR (DSe 100%, DSp 75%). Maximum likelihood latent class analysis (TAGS) | Ramilo *et al.,* 2014 |
| 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 (DSe: 69%, DSp:93%) and heart imprint (DSe: 61%, DSp: 60%). Bayesian latent class analysis | Buss *et al.,* 2019 |
| Cytology | Surveillance  | Flat oysters from three farms in South Australia (high prevalence populations 60–90%, but low intensity of infection) | Heart imprints | *Ostrea angasi* | 61% (400) | 60% (400) | Histology (DSe: 76%, DSp: 93%) and real-time PCR (DSe: 69%, DSp:93%) Bayesian latent class analysis | Buss *et al.,* 2019 |
| Surveillance | 28 flat oysters from one site in New Zealand (high prevalence 60–96%) | Heart imprints | *O. chilensis* | 59.3% (28) | 100% (28) | Combination conventional PCR and ISH (DSe & DSp: 100%) | Diggles *et al.,* 2003 |
| *In situ* hybridisation (Cochennec *et al.,* 2000) | Surveillance | 28 flat oysters from one site in New Zealand (high prevalence 60–96%) | Tissue section | *O. chilensis* | 100% (28) | 27.3% (28) | Combination heart imprint and histology (DSe & DSp: 100%) | Diggles *et al.,* 2003 |
| Conventional PCR *Bonamia* spp. (Cochennec *et al.,* 2000) | Surveillance | 28 flat oysters from one site in New Zealand (high prevalence 60–96%) | Gonad and digestive gland | *O. chilensis* | 88.2% (28) | 36.4% (28) | Combination heart imprint and histology (DSe & DSp: 100%)  | Diggles *et al.,* 2003 |
| ~~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 | 30 flat oysters from an area affected by *B. ostreae* and *B. exitiosa* in Galicia, Spain | Gills | *Ostrea edulis* | 91% (30) | 100% (30) | ISH (DSe: 82%, DSp:88%), Histology (Des 63%, DSp 88%), and real-time PCR (DSe 100%, DSp 75%) Maximum likelihood latent class analysis (TAGS)  | Ramilo *et al.,* 2014 |
| 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) | 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* spp. | 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* spp.(Corbeil *et al.,* 2006a), | 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 imprint (DSe: 61%, DSp: 60%) Bayesian latent class analysis | Buss *et al.,* 2019 |
| TaqMan real-time PCR*Bonamia* spp. | 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. exitiosa* (Ramilo *et al.,* 2013) | Surveillance | Flat oysters produced in hatchery derived from 5 origins, deployed in the field, in a *B. ostreae & B. exitiosa* endemic area (Galicia, Spain). High prevalence populations | Gills | *Ostrea edulis* (2–3 years) | 100% (137) | 77% (137) | Histology (DSe: 54%, DSp: 96%) and conventional PCR. Maximum likelihood latent class analysis (TAGS) | Ramilo *et al.,* 2013 |
| Surveillance | 30 flat oysters from an area affected by *B. ostreae* and *B. exitiosa* in Galicia, Spain | Gills | *Ostrea edulis* | 100% (30) | 75% (30) | ISH (DSe: 82%, DSp:88%), histology (DSe 63%, DSp 88%), and PCR-RFLP (DSe 91%, DSp 100%).Maximum likelihood latent class analysis, (TAGS) | Ramilo *et al.,* 2014 |

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

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\* \*

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

**NB:** First adopted in 1995 as bonamiosis. Most recent updates adopted in 2022 (Sections 2.2.1 and 2.2.2).

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