CHAPTER 2.4.2.

INFECTION WITH *BONAMIA EXITIOSA*

1. Scope

Bonamia exitiosa is a *Haplosporidia* protozoan parasite (Carnegie & Cochennec-Laureau, 2004) infecting haemocytes of several oyster species and inducing physiological disorders and eventually death of the animal (Cranfield *et al.,* 2005; Dinamani *et al.,* 1987). For the purpose of this chapter, infection with *Bonamia exitiosa* is considered to be infection with *B. exitiosa* previously named *B. exitiosus* (Berthe & Hine, 2003; Hine *et al.,* 2001). This definition excludes infections with *B. ostreae* (Pichot *et al.,* 1979), *B. roughleyi* (Cochennec *et al.,* 2003; Farley *et al.,* 1988) and *B. perspora* (Carnegie *et al.,* 2006). *Bonamia* spp. that are not identified to the species level (Burreson *et al.,* 2004; Campalans *et al.,* 2000; Kroeck & Montes, 2005) should be referred to the appropriate WOAH Reference Laboratory.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Bonamia exitiosa (Berthe & Hine, 2003; Hine *et al.,* 2001), no strain identified.

2.1.2. Survival outside the host

Currently unknown.

2.1.3. Stability of the agent (effective inactivation methods)

Currently unknown.

2.1.4. Life cycle

Transmission of the parasite directly from host to host is possible and transmission by infective stages carried passively on currents between oyster beds is suspected (Cranfield *et al.,* 2005; Hine, 1996).

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: Argentinean flat oyster (*Ostrea puelchana*), Ariake cupped oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*), Australian mud oyster (*Ostrea angasi*), Chilean flat oyster (*Ostrea chilensis*), crested oyster (*Ostrea equestris*), eastern oyster (*Crassostrea virginica*), European flat oyster (*Ostrea edulis*) and Olympia oyster (*Ostrea lurida*).

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*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: Pacific cupped oyster (*Magallana* [syn. *Crassostrea*] *gigas*) and Sydney rock oyster (*Saccostrea glomerata*).

2.2.3. Species or subpopulation predilection (probability of detection)

Ostrea chilensis and *O. angasi* (Dinamani *et al*., 1987; Hine & Jones, 1994) greater than or equal to 58 mm in length. In *O. chilensis*, mean intensity of *Bonamia* infection was significantly higher in female and spent oysters than in male and hermaphrodite oysters (Hine *et al.,* 2002).

2.2.4. Target organs and infected tissue

Bonamia exitiosa is an intrahaemocytic protozoan, but it can be observed extracellularly (Dinamani *et al*., 1987). This intrahaemocytic protozoan quickly becomes systemic and can be found in different organs, especially in the connective tissues of gills and mantle (Hine, 1991a). In *Ostrea angasi*, the parasite is epitheliotropic, and apparently very light infections may cause a massive focal haemocyte infiltration with necrotic foci. In *O. edulis*, the parasite is associated with heavy haemocytic infiltration and appears in the connective tissue of different 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. Persistent infection with lifelong carriers

Infection is often fatal depending on host and environmental conditions.

2.2.6. Vectors

None identified.

Detection of DNA from *B. exitiosa* in *Crassostrea gigas* suggests that this species may act as carrier or reservoir of *B. exitiosa* (Lynch *et al.,* 2010).

2.2.7. Known or suspected wild aquatic animal carriers

Bonamia exitiosa usually infects wild populations of susceptible species (see Section 2.2.1).

2.3. Disease pattern

2.3.1. Transmission mechanisms

In *O. chilensis* transmission of the parasite directly from host to host is possible. Released infective particles are ingested by oysters and enter the haemolymph from the gut (Hine, 1991a; 1991b). Infective particles are phagocytosed by agranular haemocytes, but they are able to resist lysis within the haemocyte (Hine & Wesney, 1994).

The parasite's DNA was detected in larvae incubated in the palleal cavity of adult oysters suggesting possible transmission between these two age groups. Larvae might thus contribute to the spread of the parasite during their planktonic life (Arzul *et al.,* 2010).

2.3.2. Prevalence

Prevalence is variable in *O. chilensis* (from 0% to nearly 80%) (Cranfield *et al.,* 2005; Diggles *et al*., 2002). 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) and salinity (40%), starvation (prolonged holding in filtered sea water), handling (vigorous stirring four times per day), or heavy infection with an apicomplexan (Hine, 2002), can affect the disease dynamics of *B. exitiosa* in *O. chilensis* (Hine *et al.,* 2002). 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.

Prevalence is variable in *O. edulis* in which co-infection with *B. ostreae* was reported (Abollo *et al.,* 2008).

2.3.3. Geographical distribution

Infection with *B. exitiosa* is found in *O. chilensis* in the Foveaux Strait and other locations around South Island, New Zealand (Dinamani *et al*., 1987; Doonan *et al.,* 1994); in *O. angasi* in Australia (Port Philip Bay, Victoria; Georges Bay, Tasmania; and Albany, Western Australia) (Corbeil *et al.,* 2006b; Hine, 1996; Hine & Jones, 1994); in *O. edulis* in Galicia (Spain) (Abollo *et al.,* 2008), in the Adriatic Sea in Italy (Narcisi et al. 2010), in the Mediterranean Sea in France and in Cornwall in the United Kingdom; and in *O. stentina* in Tunisia (Hill *et al.,* 2010).

2.3.4. Mortality and morbidity

Infection is often lethal. In *O. chilensis*, death usually occurs concurrently to the highest intensity infection level, 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).The impact of *B. exitiosa* in *O. edulis* or *O. stentina* has not been evaluated yet.

2.3.5. Environmental factors

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%) water compared with cold water (7°C for 1 hour daily) and to hyposaline water (15%) (Hine *et al.,* 2002). However, in this study, variation in temperature and salinity was used as a stressor.

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 yet.

2.4. Control and prevention

2.4.1. Vaccination

None.

2.4.2. Chemotherapy

None.

2.4.3. Immunostimulation

None.

2.4.4. Resistance breeding

None.

2.4.5. Restocking with resistant species

None.

2.4.6. Blocking agents

None.

2.4.7. Disinfection of eggs and larvae

No data available.

2.4.8. General husbandry practices

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 salinity (40%), starvation, handling, or heavy infection with other parasites, as well as decreasing density, should help to reduce the impact of the disease (Cranfield *et al.,* 2005; Hine *et al.,* 2002).

3. Sampling

3.1. Selection of individual specimens

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

Sampling should be organised once a year when prevalence is known to be at a maximum. When such data are not available in a particular ecosystem, sampling should preferably be carried out between January and April in the Southern Hemisphere (Hine, 1991a).

3.2. Preservation of samples for submission

For histology, the best preservative is Davidson's AFA, but 10% buffered formalin or other standard histology fixatives are also acceptable. For polymerase chain reaction (PCR) assays, samples must be preserved in 95–100% ethanol and not denatured alcohol.

3.3. Pooling of samples

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

3.4. Best organs or tissues

A 3–5 mm thick section of tissues including gills, mantle, gonad, and digestive gland, is used for diagnosis of *B. exitiosa* by histology. Gills and/or heart are preferred for some tests, including imprints and polymerase chain reaction (PCR).

3.5. Samples/tissues that are not suitable

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

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Clinical signs include dead or gaping oysters, but these clinical signs are not pathognomonic for infection with *B. exitiosa* and could be indicative of other infections.

4.1.2. Behavioural changes

Gaping.

4.2. Clinical methods

4.2.1. Gross pathology

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

4.2.2. Clinical chemistry

None.

4.2.3. Microscopic pathology

In *O. chilensis*, lesions occur in the connective tissue of the gill and mantle, and in the vascular sinuses around the stomach and intestine. The sub-epithelial layer of mantle connective tissue, which is infiltrated by groups of parasitised granular haemocytes, presents a dissociated appearance (Dinamani *et al*., 1987). In apparently light infections of gill and digestive diverticular epithelia in

O. angasi, faintly staining pale basophilic intracellular *B. exitiosa* results in massive epithelial hyperplasia. In *O. edulis*, the parasite was detected in the connective tissues of different organs in association with heavy haemocytic infiltration (Abollo *et al.,* 2008).

4.2.4. Wet mounts

None.

4.2.5. Imprints

Spherical or ovoid organisms (2–5 μm wide) can be observed within the haemocytes in heart or gill imprints.

4.2.6. Electron microscopy/cytopathology

In advanced infection, the parasite can be observed within the haemocytes.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

None.

4.3.1.1.2. Imprints

Samples to be taken: oyster spat or heart ventricle or gills from live hosts of 2 years of age or more.

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

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

Levels of validation:

- *Specificity and sensitivity:* low specificity, but with a sensitivity better than histological examination (Diggles *et al*., 2003). When compared with the PCR and *in-situ* hybridisation (ISH) combined, heart imprints show a sensitivity of 59.3% and a specificity of 100% in *O. chilensis* (Diggles *et al*., 2003).
- *Gold standard:* tissue imprint sensitivity is higher than histology, which is the gold standard, although it is not parasite species specific.

Interpretation of results:

- A positive result is 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 and an eosinophilic nucleus (colours may vary with the stain used) and, because they spread on the slide, they can appear larger on imprints than on histological examination.
- In susceptible species within the known geographical range of infection with *B. exitiosa,* a positive result is indicative of infection with *B. exitiosa* in *O. chilensis* and *O. angasi*, but with *B. exitiosa* or *B. ostreae* in *O. edulis.*
- In other species or outside the known geographical range of infection with *B. exitiosa*, a positive result is indicative of infection with a *Bonamia* species that needs to be confirmed by the WOAH Reference Laboratory.

Availability of commercial tests: quick staining kits are commercially available (e.g. Hemacolor®).

4.3.1.1.3. Fixed sections

4.3.1.1.3.1. Histology

Samples to be taken: live or freshly dead oysters.

Technical procedure: sections of tissue that include gills, digestive gland, mantle, and gonad should be fixed for 24 hours in Davidson's fixative or in other standard histology fixatives including 10% buffered formalin, followed by normal processing for paraffin histology and staining, for example, with haematoxylin and eosin. Observations are made at increasing magnifications up to ×1000.

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

Levels of validation:

- *Specificity and sensitivity:* in *O. chilensis*, low specificity but sensitivity is good for moderate- to high-intensity infections, and low for low-intensity infections. When compared with the PCR and the ISH combined, histology shows a sensitivity of 44% (lower than heart imprints) and a specificity of 100% (Diggles *et al*., 2003).
- *Gold standard:* histology is the gold standard and is the recommended surveillance method in regions only infected by *B. exitiosa*. However in regions where *B. exitiosa* and *B. ostreae* are sympatric, a positive result by histology needs to be confirmed by molecular characterisation.

Interpretation of results:

- A positive result is the presence of parasites as very small cells of 2–5 μm wide within the haemocytes or free in the connective tissue or sinuses of the gill, gut and mantle epithelium in *O. chilensis*. They are often associated with an intense disseminated haemocyte infiltration in *O. chilensis* but intense focal haemocyte infiltration in *O. angasi*. To avoid any doubt, the parasite has to be observed inside the haemocyte for a positive diagnosis. The technique is not species specific.
- In susceptible species within the known geographical range of infection with *B. exitiosa* a positive result is indicative of infection with *B. exitiosa* in *O. chilensis* and *O. angasi*, but with *B. exitiosa* or *B. ostreae* in *O. edulis*.
- In other species or outside the known geographical range of infection with *B. exitiosa*, a positive result is indicative of infection with a *Bonamia* species that needs to be confirmed by the WOAH Reference Laboratory.

Availability of commercial tests: no commercially available tests.

4.3.1.1.3.2. Transmission electron microscopy

Samples to be taken: live or freshly dead oysters.

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

Positive controls: none.

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

Levels of validation:

- *Specificity and sensitivity:* better specificity than imprints and histology. Transmission electron microscopy (TEM) permits the differentiation of *B. exitiosa* from other closely related microcells, such as *B. ostreae*.

Interpretation of results:

- A positive result is the presence of parasites within the haemocytes. In *O. chilensis*, four parasite development stages have been described in infected oysters corresponding to dense forms (Stage 1), intermediate forms (Stage 2), plasmodial forms (Stage 3) and vacuolated forms (Stage 4) (Hine, 1991b; Hine *et al.,* 2001). Intracellular structures include mitochondria, haplosporosomes, Golgi apparatus and persistent intranuclear microtubules.
- Unlike other haplosporidians, including *B. ostreae*, a stage containing a large vacuole derived from enlargement of one or more mitochondria has been observed in *B. exitiosa* (Hine, 1991b; Hine *et al.,* 2001). Dense forms of *B. exitiosa* are slightly larger in size (3 ± 0.3 μm mean diameter number of parasites = 61) in comparison with *B. ostreae*, (2.4 ± 0.5 μm mean diameter number of parasites = 64) and have more haplosporosomes, mitochondrial profiles and lipoid bodies per ultrastructure section, as well as smaller tubulo-vesicular mitochondria. In addition, dense, but not light, forms of *B. ostreae* lack nuclear membrane-bound Golgi/nuclear cup complexes and a vacuolated stage (Hine *et al.,* 2001).

Availability of commercial tests: no commercially available tests.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Not available.

4.3.1.2.2. Antibody-based antigen detection methods

Not currently available, but one of the two monoclonal antibodies raised against the cell membrane of *B. ostreae* reacted with *B. exitiosa* (Mialhe *et al.,* 1988).

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. Polymerase chain reaction (PCR)

Samples to be taken: live or freshly dead oysters.

Technical procedure: tissue samples are placed in 95–100% ethanol or frozen until DNA is extracted. DNA extraction is accomplished by proteinase K digestion overnight at 50°C, and phenol–chloroform extraction with ethanol precipitation (Cochennec *et al.,* 2000).

A PCR protocol developed for the detection of *B. ostreae* has been shown to allow *B. exitiosa* detection (Carnegie & Cochennec-Laureau, 2004; Diggles *et al*., 2003; Hine *et al.,* 2001). The primer pair Bo–Boas (5'-CAT-TTA-ATT-GGT-CGG-GCC-GC-3' and 5'-CTG-ATC-GTC-TTC-GAT-CCC-CC-3', respectively) amplifies a 304 bp product from the small subunit (SSU) rDNA region (Cochennec *et al.,* 2000). PCR mixtures contain buffer (500 mM KCl, 100 mM Tris/HCl [pH 9.0 at 25°C] and 1% Triton® X-100), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 μM forward and reverse primers, 0.02 units μl^{-1} Taq DNA polymerase, and 0.2 ng μl^{-1} of the DNA template in a total volume of 50 μl. Samples are denatured in a thermocycler for 5 minutes at 94°C before being submitted to 30 cycles (94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute), followed by a final extension of 10 minutes at 72°C.

A second primer pair C_F and C_R (5'-CGG-GGG-CAT-AAT-TCA-GGA-AC-3' and 5'-CCA-TCT-GCT-GGA-GAC-ACA-G-3', respectively), also designed to amplify a 760 bp product from the SSU rDNA of *B. ostreae*, should amplify *B. exitiosa* (Carnegie & Cochennec-Laureau, 2004).

A TaqMan PCR assay using primers and probe targeting the ITS1 (internal transcribed spacer) region can also be used for *Bonamia* spp. detection (Corbeil *et al.,* 2006a). Sensitivity is good; this assay does not amplify *Haplosporidium nelsoni*, *H. costale* or *Mikrocytos mackini*. However, it has not yet been thoroughly validated.

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

Levels of validation:

- *Specificity and sensitivity:* based on target DNA sequence similarity, the primer pair Bo–Boas should amplify all microcell haplosporidians (Carnegie & Cochennec-Laureau, 2004). The sensitivity of the assay is higher than histocytological methods but lower than *in-situ* hybridisation (Diggles *et al*., 2003).
- *Gold standard:* when compared with histology and heart imprints combined, and assuming that these latter techniques present 100% sensitivity and specificity, PCR shows a sensitivity of 88.2% (lower than ISH) and a specificity of 36.4% (Diggles *et al*., 2003).

Interpretation of results:

- A positive result is an amplicon of the appropriate size, with all negative controls negative and all positive controls positive.
- The PCR assay is not species specific. The sequence of the SSU rDNA gene from *Bonamia exitiosa* shows polymorphism with that of *B. ostreae* and *B. roughleyi* by restriction fragment length polymorphism (RFLP) analysis after digesting Bo–Boas PCR products with *Bgl* I and *Hae* II. *Bonamia ostreae* and *B. exitiosa* show the same profile (two products of 115 and 189 bp) when digested with *Hae* II, while the *B. roughleyi* PCR product is not digested. The B. ostreae profile consists of two bands of 120 and 180 bp when digested with *Bgl* I, while B. exitiosa and *B. roughleyi* PCR products are not digested (Carnegie & Cochennec-Laureau, 2004; Hine *et al.,* 2001). However, based on sequence analysis, the PCR-RFLP does not distinguish *B. exitiosa* and other related isolates from *B. perspora*.
- In *O. chilensis* and *O. angasi* from New Zealand and Australia, respectively, an expected PCR-RFLP result, associated with a positive result by means of histology or imprints, is confirmatory for infection with *B. exitiosa;*
- In other species, or outside the known geographical range of infection with *B. exitiosa*, an expected PCR-RFLP result, associated with a positive result by means of histology or imprints, is strongly indicative of infection with *B. exitiosa,* but PCR product sequencing and, if possible, TEM is necessary before confirmatory diagnosis.

Availability of commercial tests: no commercially available tests.

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

Samples to be taken: live or freshly dead oysters.

Technical procedure: an ISH protocol developed for the detection of *B. ostreae* has been shown to allow *B. exitiosa* detection (Cochennec *et al.,* 2000; Diggles *et al*., 2003). This assay uses a 300 bp digoxigenin-labelled probe targeting the SSU rDNA gene. Tissue samples are placed in Davidson's fixative for 24 hours and are then embedded in paraffin. Sections of 5 μm are cut, placed on silane-coated slides and then baked overnight in an oven at 60°C. After de-waxing, slides are treated with proteinase K (100 μg ml^{-1}) in TE buffer (50 mM Tris, 10 mM EDTA [ethylene diamine tetra-acetic acid]) at 37°C for 30 minutes. 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 NaCi, 600 mM NaCl, pH 7], 50% formamide, 1 × Denhardt's solution, 250 μg ml^{-1} yeast tRNA, 10% dextran sulphate) containing 20 ng of the digoxigenin-labelled probe. After denaturation for 5 minutes at 95°C, hybridisation is performed by incubating the slides in a humid chamber overnight at 42°C. The probe is produced by PCR using the previously described primer pair Bo–Boas with digoxigenin incorporation. 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.

Another ISH protocol uses a cocktail of three 5' digoxigenin-labelled probes specific to closely related members of the *B. exitiosa–B. roughleyi* group (Hill *et al.,* 2010). However, this assay has not been thoroughly validated.

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

Levels of validation:

- *Specificity and sensitivity:* specificity is higher than histocytological methods. However, the Bo–Boas probe is also able to detect *Haplosporidium nelsoni* in *Crassostrea virginica, B. ostreae* in *O. edulis* but not *Mikrocytos mackini* in *C. gigas* (Carnegie & Cochennec-Laureau, 2004; Cochennec *et al.,* 2000). Sensitivity of the assay is higher than histocytological methods and PCR (Diggles *et al*., 2003).
- *Gold standard:* when compared with histology and heart imprints combined, and assuming that these latter techniques present 100% sensitivity and specificity, ISH shows a sensitivity of 100% (higher than PCR) and a specificity of 27.3% (Diggles *et al*., 2003).

Interpretation of results:

- A positive result corresponds to dark labelled parasites inside the haemocytes, with all negative controls negative and all positive controls positive.
- In susceptible species, within the known geographical range of infection with *B. exitiosa* a positive result is indicative of infection with *B. exitiosa* in *O. chilensis* and *O. angasi*, but with *B. exitiosa* or *B. ostreae* in *O. edulis*
- In other species or outside the known geographical range of infection with *B. exitiosa*, a positive result is indicative of infection with a *Bonamia* species that needs to be confirmed by the WOAH Reference Laboratory.

Availability of commercial tests: DIG nucleic acid detection kit (Boehringer Mannheim).

4.3.1.2.3.3. Sequencing

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

4.3.1.2.4. Agent purification

None.

4.3.2. Serological methods

None applicable.

5. Rating of tests against purpose of use

As an example, the methods currently available for targeted surveillance and diagnosis of infection with *B. exitiosa* are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	c	d
Tissue imprints	d	d	a	a	a	c
Histopathology	d	d	a	a	a	c
Transmission EM	d	d	d	d	d	b
***In-situ* DNA probes**	d	d	d	d	d	b
PCR and TaqMan PCR	a	a	a	a	a	c
PCR-RFLP	d	d	d	d	d	b
Sequence	d	d	d	d	d	a

PLs = postlarvae; EM = electron microscopy; PCR = polymerase chain reaction;
RFLP = restriction fragment length polymorphism.

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

Prescribed methods for targeted surveillance to declare freedom from infection as outlined in the *Aquatic Code* are: tissue imprints (heart or gills), histology or PCR in regions only infected by *B. exitiosa*. However in regions where *B. exitiosa* and *B. o*streae are sympatric, a positive result by histology needs to be confirmed by molecular characterisation.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

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

7.2. Definition of confirmed case

In susceptible species within the known geographical range of infection with *B. exitiosa*, a confirmed case of *B. exitiosa* is a positive result by tissue imprints, histology or *in-situ* hybridisation combined with a positive result by PCR-RFLP and sequencing.

In other host species or outside the known range of *B. exitiosa*, TEM confirmation is recommended. However, this technique is only suitable for samples that have high intensities of infection.

8. References

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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 2023 (SECTIONS 2.2.1 AND 2.2.2).