CHAPTER 2.4.3.

INFECTION WITH *BONAMIA OSTREAE*

1. Scope

Bonamia ostreae is a *Haplosporidia* protozoan parasite (Carnegie & Cochennec-Laureau, 2004; Lopez-Flores *et al.,* 2007) infecting haemocytes of flat oysters, *Ostrea edulis,* and inducing physiological disorders and eventually death of the animal (Grizel, 1985). For the purpose of this chapter, infection with *Bonamia ostreae* is considered to be infection with *B. ostreae*. This definition excludes infections with *B. exitiosa* (Hine *et al*., 2001), *B. roughleyi* (Cochennec *et al*., 2003) and *B. perspora* (Carnegie *et al.,* 2006). *Bonamia* spp. that are not identified to the species level should be referred to the appropriate WOAH Reference Laboratory.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Bonamia ostreae (Pichot *et al*., 1979), no strain identified.

2.1.2. Survival outside the host

Up to 58% of parasites purified from highly infected oysters seem to survive after 1 week in seabed bore water at 15°C (Arzul *et al.,* 2009).

2.1.3. Stability of the agent (effective inactivation methods)

Peracetic acid bath (0.001% and 0.005%) has been shown to reduce contamination of oysters by *B. ostreae* (Grizel, 1985).

2.1.4. Life cycle

The life cycle outside the host is unknown but transmission of the parasite directly from host to host by cohabitation or by inoculation of purified parasites is possible (Hervio *et al.,* 1995), suggesting that no intermediate host is needed.

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: Ariake cupped oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*), European flat oyster (*Ostrea edulis*) and Chilean flat oyster (*Ostrea chilensis*).

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: Argentinean flat oyster (*Ostrea puelchana*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: beadlet anemone (*Actina equina*), brittle star (*Ophiothrix fragilis*), European sea squirt (*Ascidiella aspersa*), grouped zooplankton and Pacific cupped oyster (*Magallana* [syn. *Crassostrea*] *gigas*).

2.2.3. Species or subpopulation predilection (probability of detection)

Ostrea edulis is the only known natural susceptible species and infection intensity increases concurrently to mortality with age and/or size of the oysters (Culloty & Mulcahy, 1996; Grizel, 1985).

2.2.4. Target organs and infected tissue

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. Persistent infection with lifelong carriers

Infection is often fatal depending on host and environmental conditions.

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).

PCR positive signal observed in *Crassostrea gigas* suggests that this species may act as a carrier or reservoir of *B. ostreae* (Lynch *et al.,* 2010).

2.2.7. Known or suspected wild aquatic animal carriers

Wild populations of flat oysters *Ostrea edulis* are also infected by *B. ostreae*.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Direct transmission from host to host is possible. The infective form and ways of entry and release remain undetermined. The parasite was observed in larvae incubated in the pallial 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). A lag time of at least 3 months is generally observed before detecting the parasite in disease free batches moved into infected areas.

2.3.2. Prevalence

Prevalence is variable (from 0% to 80%). Prevalence 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; Grizel, 1985; Engelsma *et al.,* 2010).

2.3.3. Geographical distribution

Infection with *B. ostreae* has been found in Europe (France, Ireland, Italy, Netherlands, Portugal, Spain and the United Kingdom), Canada (British Columbia) and the United States of America (California, Maine and Washington States) (Carnegie & Cochennec-Laureau, 2004).

2.3.4. Mortality and morbidity

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

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 psu) 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 are reported (Arzul *et al.,* 2006; Culloty & Mulcahy, 1996). 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)

2.4. Control and prevention

2.4.1. Vaccination

None.

2.4.2. Chemotherapy

None.

2.4.3. Immunostimulation

None.

2.4.4. Resistance breeding

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

2.4.5. Restocking with resistant species

Resistant strains of *Ostrea edulis* developed through selective breeding may offer an alternative in infected areas.

2.4.6. Blocking agents

None.

2.4.7. Disinfection of eggs and larvae

No data available.

2.4.8. General husbandry practices

Mortalities caused by bonamiosis can be reduced using suspension culture, lower stocking densities or by culturing *Ostrea edulis* with *Crassostrea gigas,* which are not naturally susceptible to infection (Carnegie & Cochennec-Laureau, 2004). Oyster seed from hatcheries are preferred to seed from natural settlements as the latter appear to be significantly more parasitised (Conchas *et al*., 2003).

3. Sampling

3.1. Selection of individual specimens

Gaping or freshly dead individuals (2 or more years old) should be sampled by priority, to increase the chances of finding infected oysters. For histology, only live (including moribund) oysters 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 in late winter-early spring or in autumn (Arzul *et al.,* 2006; Culloty & Mulcahy, 1996; Engelsma *et al.,* 2010).

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 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. ostreae* by histology. Gills and/or heart are preferred for some tests, including imprints and 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. ostreae* and could be indicative of other infections.

4.1.2. Behavioural changes

Gaping.

4.2. Clinical methods

4.2.1. 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.

4.2.2. Clinical chemistry

None.

4.2.3. Microscopic pathology

Dense infiltrations of haemocytes, some containing parasites, in the connective tissue of the gill and mantle, and in the vascular sinuses around the stomach and intestine can be seen in fixed sections (Comps *et al*., 1980).

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

Not applicable.

4.3.1.1.2. Imprints

Samples to be taken: soft tissues from oyster spat, and heart ventricle or gills from live hosts that are 2 years or older.

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 (Da Silva & Villalba, 2004). However, it would appear that the heart imprint technique is not reliable for detecting latent infections.
- *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 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 wider on imprints than on histological examination. Multinucleated cells can be observed. The technique is not parasite species specific.
- In susceptible species within the known geographical range of infection with *B. ostreae* a positive result is strongly indicative of infection with *B. ostreae* or *B. exitiosa*.
- In other species or outside the known geographical range of infection with *B. ostreae*, 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 to ×1000.

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

Levels of validation:

- *Specificity and sensitivity:* low specificity but sensitivity is good for moderate- to high-intensity infections, and low for low-intensity infections.
- *Gold standard:* histology is the gold standard and is the recommended surveillance method in regions only infected by *B. ostreae*. 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 gill, gut and mantle epithelium, often associated with an intense inflammatory reaction. 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. ostreae* a positive result is strongly indicative of infection with *B. ostreae* or *B. exitiosa*.
- In other species or outside the known geographical range of infection with *B. ostreae*, 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 citratee.

Positive controls: none.

Levels of validation:

- *Specificity and sensitivity:* better specificity than imprints and histology. Transmission electron microscopy (TEM) may help to differentiate *B. ostreae* from other closely related microcells, such as *B. exitiosa*.

Interpretation of results:

- A positive result is the presence of parasites within the haemocytes. Different stages, including uninucleate, diplocaryotic 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.
- Dense forms of *B. ostreae* are more dense and slightly smaller in size (2.4 ± 0.5 μm mean diameter (number of parasites = 64) compared with *B. exitiosa* (3 ± 0.3 μm mean diameter (number of parasites =61) and have fewer haplosporosomes, mitochondrial profiles and lipoid bodies per ultrastructure section, as well as larger tubulo-vesicular mitochondria than *B. exitiosa*. In addition, dense 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.

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*.

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

An immunofluorescent technique based on monoclonal antibodies was developed and had sensitivity similar to tissue imprints. However, this technique gave unclear results when tested extensively on oysters from Maine, USA (Carnegie & Cochennec-Laureau, 2004). Although direct monoclonal antibody sandwich immunoassay for the detection of *B. ostreae* in haemolymph samples of *O. edulis* was developed (Cochennec *et al*., 1992) and marketed commercially for a few years in the mid-1990s, it is no longer available on the market. The specificity and sensitivity of this latter technique compared with histology were 76.7% and 106%, respectively (Cochennec *et al*., 1992).

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 50–55°C, and phenol–chloroform extraction with ethanol precipitation (Carnegie *et al.,* 2000; Cochennec *et al*., 2000) or the spin-column methodology using commercially available kits (e.g. QIAGEN) (Carnegie *et al.,* 2000).

Three conventional PCR protocols with three different primer pairs targeting the small subunit (SSU) rDNA have been developed for *Bonamia ostreae:*

The first primer pair is 5'-CAT-TTA-ATT-GGT-CGG-GCC-GC-3' and 5'-CTG-ATC-GTC-TTC-GAT-CCC-CC-3', designated Bo and Boas respectively, and amplifies a 300 bp product (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.

The second primer pair is 5'-CGG-GGG-CAT-AAT-TCA-GGA-AC-3' and 5'-CCA-TCT-GCT-GGA-GAC-ACA-G-3', designated CF and CR, respectively, and amplifies a 760 bp product (Carnegie *et al.,* 2000). PCR reaction mixtures contain buffer (200 mM Tris/HCl [pH 8.4], 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.05 µM forward and reverse primers, 0.05 units µl–1 Taq DNA polymerase, and 1 ng μl^{-1} of the DNA template in a total volume of 50 µl. Samples are submitted to 35 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.

The third primer pair is 3'-CAA-TGG-TGC-GTT-CAA-CGA-T-5' and 3'-GGG-TTC-GCG-GTT-GAA-TTT-TA-5', designated BoosF03 and BoosR03, respectively, which amplifies a 352 bp product (Engelsma *et al.,* 2010). PCR reaction mixtures contain buffer (1×), 2 mM $MgCl_2$ 0.2 mM of each dNTP, 0.4 µM of each primer, 2 units Taq DNA polymerase in distilled water with 0.005% (v/v) Nonidet P-40 and 2 µl of the DNA template in a total volume of 50 µl. Samples are denatured in a thermocycler for 2 minutes at 94°C before being submitted to 40 cycles (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 45 seconds) followed by a final extension of 7 minutes at 72°C.

Two TaqMan PCR assays can also be used:

A TaqMan PCR assay using primers and probe targeting the ITS1 (internal transcribed spacer) detects *Bonamia* spp. detection (Corbeil *et al.,* 2006). Sensitivity was good and this assay did not amplify *Haplosporidium nelsoni*, *H. costale* or *Mikrocytos mackini*. However, this assay was not thoroughly validated.

Another TaqMan PCR assay using primers and probe that targets a small region (67 bp) of the small subunit (SSU) rDNA has also developed for *B. ostreae* (Marty *et al.,* 2006). Primers and probe were

designed to be specific for *Bonamia* spp. and do not amplify other *Haplosporidia*. Sensitivity and specificity are good and higher than histopathology.

Lastly an SYBR® Green real-time PCR assay has been developed to detect and quantify *B. ostreae* (Robert *et al.,* 2009). This assay targets a 201 bp region of the actin 1 gene of the parasite. This assay has been shown to only target *B. ostreae* and not closely related parasites including *B. exitiosa*. The minimum detection limit was estimated at 50 gene copies and the assay appeared to be at least ten-times more sensitive than the conventional PCR. A good correlation was observed between semi-quantification of the parasite by heart imprint and this real-time PCR assay.

Positive/negative controls: these are compulsory. Positive controls are: 1) PCR with specific primers to genomic DNA from a highly infected host or DNA from purified parasites; 2) non-specific 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 first conventional assay (Cochennec *et al.,* 2000) should amplify all microcell haplosporidians, and the second one (Carnegie *et al.,* 2000) should at least amplify *B. ostreae* and *B. exitiosa* (Carnegie & Cochennec-Laureau, 2004); the third one apparently only amplifies *B. ostreae* (Engelsma *et al.,* 2010). The sensitivity of these assays is higher than histocytological methods. The two TaqMan PCR assays detect *Bonamia* spp. but not other *Haplosporidia*. The SYBR® Green real-time PCR assay only detects *B. ostreae.*
- *Gold standard:* the sensitivity and specificity of the first conventional PCR assay (Cochennec *et al.*, 2000) were calculated against histocytological methods (histology and gill imprints) and were shown to be 92% and 87%, respectively. The sensitivity and specificity of histocytological methods (histology and gill imprints) were calculated against the first conventional PCR assay (Cochennec *et al.,* 2000) and were shown to be 66% and 97%, respectively (Balseiro *et al*., 2006). Sensitivity and specificity have also been evaluated for the second TaqMan PCR assay and initially estimated at 88% and 99%, respectively.

Interpretation of results:

- A positive result is an amplicon of the appropriate size, with all negative controls negative and all positive controls positive.
- Neither assay is species specific. The sequence of the SSU rDNA gene of *B. ostreae* shows polymorphism with that of *B. exitiosa, B. roughleyi* or *B. perspora* by restriction fragment length polymorphism (RFLP) analysis after digesting the PCR product Bo–Boas with *Hae* II and *Bgl* I. The obtained profiles vary according to the parasite species. *Bonamia ostreae, B. perspora* and *B. exitiosa* show the same profile (two products of 115 and 189 bp) when digested with *Hae* II, while the *B. roughleyi* 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, B. perspora* and *B. roughleyi* are not digested (Cochennec *et al*., 2003; Hine *et al*., 2001).
- In susceptible species within the known geographical range of infection with *B. ostreae*, an expected PCR-RFLP result, associated with a positive result by means of histology or imprints, is confirmatory for infection with *B. ostreae.*
- In other species or outside the known geographical range of infection with *B. ostreae*, an expected PCR-RFLP result, associated with a positive result by means of histology or imprints, is strongly indicative of infection with *B. ostreae,* 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: two ISH protocols have been developed. The first one (Cochennec *et al*., 2000) uses a 300 bp digoxigenin-labelled probe and the second one (Carnegie *et al.,* 2003) uses three fluorescein-labelled oligonucleotide probes. All these probes target 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 50–60°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 in the first protocol or in PBS (phosphate-buffered saline) buffer (150 mM NaCl, 12.5 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.2) for 15 minutes at 37°C in the second protocol.

- In the first protocol, 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 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.

- In the second protocol, 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. Probes consist of a cocktail of oligo-fluoroscein-labelled probes specific for *B. ostreae:* UME-BO-1 (5'-CGA-GGC-AGG-GTT-TGT-3'); UME-BO-2 (5'-GGG-TCA-AAC-TCG-TTG-AAC-3') and UME-BO-3 (5'-CGC-TCT-TAT-CCA-CCT-AAT-3').

Positive/negative controls: these are compulsory. Positive controls are: 1) ISH on infected host; 2) non-specific 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 and sensitivity are higher than histological examination. However, the probe Bo–Boas is able to detect *Haplosporidium nelsoni* in *Crassostrea virginica, B. exitiosa* in *O. chilensis,* but not *Mikrocytos mackini* in *C. gigas* (Cochennec *et al*., 2000). The specificity of the oligoprobe cocktail UME-BO-1, 2 and 3 has been tested and proven against *H. nelsoni* (Carnegie *et al.,* 2003), but this ISH assay probably detects other microcells including *B. exitiosa* (Carnegie & Cochennec-Laureau, 2004).
- *Gold standard:* ISH has not yet been validated against histology.

Interpretation of results:

- A positive result corresponds to labelled parasites inside the haemocytes, with all negative controls negative and all positive controls positive. In the first described protocol, they appear as dark spots, whereas in the second protocol, they correspond to small green rings, representing green fluorescence surrounding an eccentric dark region.
- In susceptible species within the known geographical range of infection with *B. ostreae* a positive result is strongly indicative of infection with *B. ostreae* or *B. exitiosa.*

- In other species or outside the known geographical range of infection with *B. ostreae*, 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) for the first protocol.

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

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.

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. ostreae* 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	b	c
Transmission EM	d	d	d	d	d	a
***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
SYBR® green real-time PCR	a	a	a	a	a	c
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 ostreae*

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. ostreae*. 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. ostreae*, a confirmed case of *B. ostreae* is a positive result by tissue imprints, histology or *in-situ* hybridisation combined with a positive result by PCR-RFLP and sequencing or SYBR® Green real-time PCR.

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

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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 2023 (SECTIONS 2.2.1 AND 2.2.2).