CHAPTER 2.4.3.

INFECTION WITH *BONAMIA OSTREAE*

1. Scope[1]

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 OIE Reference Laboratory.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

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 factor

2.2.1. Susceptible host species

Natural host: European flat oysters, *Ostrea edulis*.

Oyster species infected when moved into *B. ostreae* endemic zones: *Ostrea puelchana*, *O. angasi*, *O. chilensis* (= *Tiostrea chilensis*, *T. lutaria*) (Carnegie & Cochennec-Laureau, 2004). However, the parasite was not identified to the species level in these hosts.

Experimental assays have indicated a low infectivity of *B. ostreae* to *Crasssotrea ariakensis* (Audemard et al., 2005).

It has been speculated that *Ostrea conchaphila* (= *O. lurida*) and *Crassostrea angulata* have been infected with *B. ostreae* (Carnegie & Cochennec-Laureau, 2004), but confirmatory diagnosis has not been achieved.

Experimental work showed that the following species are not susceptible to *B. ostreae*: *C. gigas*, *Ruditapes decussatus*, *R. philippinarum*, *Mytilus edulis*, *M. galloprovincialis* (Culloty et al., 1999).

1 NB: Version adopted by the World Assembly of Delegates of the OIE in May 2012.

2.2.2. Susceptible stages of the host

Both 0+ and 1+ year-old *O. edulis* are susceptible to infection and can develop a high prevalence and high intensity of infection and even mortality over a 6-month period (Lynch et al., 2005). However, individuals older than 2 years appear to be more susceptible to the disease (Culloty & Mulcahy, 1996; Grizel, 1985; Engelsma et al., 2010). Seed from natural settlements appear to be significantly more parasitised than oyster seed from hatcheries (Conchas et al., 2003).

It has recently been shown that larvae can be infected with *B. ostreae* (Arzul et al., 2010).

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).

Polymerase chain reaction (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 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. 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

4.3.1.1.2.1. Samples to be taken

Soft tissues from oyster spat, and heart ventricle or gills from live hosts that are 2 years or older.

4.3.1.1.2.2. 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.

4.3.1.1.2.3. Positive controls

Recommended and available from the OIE Reference Laboratory.

4.3.1.1.2.4. Levels of validation

4.3.1.1.2.4.1. 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.

4.3.1.1.2.4.2. Gold standard

Tissue imprint sensitivity is higher than histology, which is the gold standard, although it is not parasite species specific.

4.3.1.1.2.5. 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 OIE Reference Laboratory.

4.3.1.1.2.6. 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

4.3.1.1.3.1.1. Samples to be taken

Live or freshly dead oysters.

4.3.1.1.3.1.2. 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.

4.3.1.1.3.1.3. Positive controls

These are recommended and available from the OIE Reference Laboratory.

4.3.1.1.3.1.4. Levels of validation

4.3.1.1.3.1.4.1. Specificity and sensitivity

Low specificity but sensitivity is good for moderate- to high-intensity infections, and low for low-intensity infections.

4.3.1.1.3.1.4.2. 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.

4.3.1.1.3.1.5. 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 OIE Reference Laboratory.

4.3.1.1.3.1.6. Availability of commercial tests

No commercially available tests.

4.3.1.1.3.2. Transmission electron microscopy

4.3.1.1.3.2.1. Samples to be taken

Live or freshly dead oysters.

4.3.1.1.3.2.2. 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. 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.

4.3.1.1.3.2.3. Positive controls

None.

4.3.1.1.3.2.4. Levels of validation

4.3.1.1.3.2.4.1. 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*.

4.3.1.1.3.2.5. 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).

4.3.1.1.3.2.6. 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

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)

4.3.1.2.3.1.1. Samples to be taken

Live or freshly dead oysters.

4.3.1.2.3.1.2. 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 C_F and C_R, 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.

4.3.1.2.3.1.3. 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) 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 OIE Reference Laboratory.

4.3.1.2.3.1.4. Levels of validation

4.3.1.2.3.1.4.1. 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*.

4.3.1.2.3.1.4.2. 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.

4.3.1.2.3.1.5. 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.

4.3.1.2.3.1.6. Availability of commercial tests

No commercially available tests.

4.3.1.2.3.2. In-situ hybridisation (ISH)

4.3.1.2.3.2.1. Samples to be taken

Live or freshly dead oysters.

4.3.1.2.3.2.2. 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').

4.3.1.2.3.2.3. 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 OIE Reference Laboratory.

4.3.1.2.3.2.4. Levels of validation

4.3.1.2.3.2.4.1. 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).

4.3.1.2.3.2.4.2. Gold standard

ISH has not yet been validated against histology.

4.3.1.2.3.2.5. 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 OIE Reference Laboratory.

4.3.1.2.3.2.6. 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 sequences are available in public gene banks, it is recommended to refer such cases to the appropriate OIE Reference Laboratory.

4.3.1.2.3.4. 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
Hispathology	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. ostreae* 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.

8. References

ARZUL I., GAGNAIRE B., BOND C., CHOLLET B., MORGA B., FERRAND S., ROBERT M. & RENAULT T. (2009). Effects of temperature and salinity on the survival of *Bonamia ostreae*, a parasite infecting flat oysters *Ostrea edulis*. *Dis. Aquat. Org.*, **85**, 67–75.

ARZUL I., LANGLADE A., CHOLLET B., ROBERT M., FERRAND S., OMNES E., LEROND S., COURALEAU Y., JOLY J.-P., FRANÇOIS C. & GARCIA C. (2011). Can the protozoan parasite *Bonamia ostreae* infect larvae of flat oysters *Ostrea edulis*? *Vet. Parasitol.*, **179 (1–3)**, 69–76.

ARZUL I., MIOSSEC L., BLANCHET E., GARCIA C., FRANÇOIS C. & JOLY J.-P. (2006). *Bonamia ostreae* and *Ostrea edulis*: a stable host-parasite system in France? Symposia proceedings, *ISVEE conference XI*, Cairns, Australia, 6–11 August 2006, 5 pp.

AUDEMARD C., CARNEGIE R., STOKES N.A., BURRESON E. & BISHOP M. (2005). Salinity effects on the susceptibility to and persistence of *Bonamia ostreae* and *Bonamia*sp. in *Crassostrea ariakensis*. *J. Shellfish Res.*, **24**, 639.

BALSEIRO P., CONCHAS R.F., MONTES J., GOMEZ-LEON J., NOVOA B. & FIGUERAS A. (2006). Comparison of diagnosis techniques for the protozoan parasite *Bonamia ostreae* in flat oyster *Ostrea edulis*. *Aquaculture*, **261**, 1135–1143.

CARNEGIE R., BARBER B.J., CULLOTY S.C., FIGUERAS A.J. & DISTEL D.L. (2000). Development of a PCR assay for detection of the oyster pathogen *Bonamia ostreae* and support for its inclusion in the *Haplosporidia*. *Dis. Aquat. Org.*, **42**, 199–206.

CARNEGIE R.B., BARBER B.J. & DISTEL D.L. (2003). Detection of the oyster parasite *Bonamia ostreae* by fluorescent *in situ* hybridization. *Dis. Aquat. Org.*, **55**, 247–252.

CARNEGIE R.B. & COCHENNEC-LAUREAU N. (2004). Microcell parasites of oysters: Recent insights and future trends. *Aquat. Living Resour.*, **17**, 519–528.

CARNEGIE R.B., BURRESON E.M., HINE P.M., STOKES N.A., AUDEMARD C., BISHOP M.J. & PETERSON C.H. (2006). *Bonamia perspora* n. sp. (*Haplosporidia*), a parasite of the oyster *Ostreaola equestris*, is the first *Bonamia* species known to produce spores. *J. Eukaryot. Microbiol.*, **53**, 232–245.

COCHENNEC N., HERVIO D., PANATIER B., BOULO V., MIALHE E., ROGIER H., GRIZEL H. & PAOLUCCI F. (1992). A direct monoclonal antibody sandwich immunoassay for detection of *Bonamia ostreae* (*Ascetospora*) in hemolymph samples of the flat oyster *Ostrea edulis* (*Mollusca: Bivalvia*). *Dis. Aquat. Org.*, **12**, 129–134.

COCHENNEC N., LE ROUX F., BERTHE F. & GERARD A. (2000). Detection of *Bonamia ostreae* based on small subunit ribosomal probe. *J. Invertebr. Pathol.*, **76**, 26–32.

COCHENNEC N., REECE K.S., BERTHE F.C.J. & HINE P.M. (2003). Revisiting *Mikrocytos roughleyi* taxonomic affiliation points to the genus *Bonamia* (*Haplosporidia*). *Dis. Aquat. Org.*, **54**, 209–217.

COMPS M., TIGÉ G. & GRIZEL H. (1980). COMPS M., TIGÉ G. & GRIZEL H. (1980). Etude ultrastructurale d'un protiste parasite de l'huître *Ostrea edulis* L. *C. R. Hebd. Acad. Sc. Paris (Sér. D)*, **290**, 383–385.

CONCHAS R.F., SANTAMARINA J., LAMA A., LONGA M.A. & MONTES J. (2003). Evolution of bonamiosis in Galicia (NW Spain). *Bull. Eur. Assoc. Fish Pathol.*, **23**, 265–272.

CORBEIL S., ARZUL I., DIGGLES B., HEASMAN M., CHOLLET B., BERTHE F.C. & CRANE M.S. (2006). Development of a TaqMan PCR assay for the detection of *Bonamia* species. *Dis. Aquat. Org.*, **71**, 75–80.

CULLOTY S.C. & MULCAHY M.F. (1996). Season-, age-, and sex-related variation in the prevalence of bonamiosis in flat oysters (*Ostrea edulis* L.) on the south coast of Ireland. *Aquaculture*, **144**, 53–63.

CULLOTY S.C., NOVOA B., PERNAS M., LONGSHAW M., MULCAHY M.F., FEIST S.W. & FIGUERAS A. (1999). Suspectibility of a number of bivalve species to the protozoan parasite *Bonamia ostreae* and their ability to act as vectors for this parasite. *Dis. Aquat. Org.*, **37**, 73–80.

DA SILVA P.M. & VILLALBA A. (2004). Comparison of light microscopic techniques for the diagnosis of the infection of the European flat oyster *Ostrea edulis* by the protozoan *Bonamia ostreae*. *J. Invertebr. Pathol.*, **85**, 97–104.

ENGELSMA M.Y., KERKHOFF S., ROOZENBURG I., HAENEN O.L.M., VAN GOOL A., SISTERMANS W., WIJNHOVEN S. & HUMMEL H. (2010). Epidemiology of *Bonamia ostreae* infecting European flat oyster *Ostrea edulis* from Lake Grevelingen, The Netherlands. *Marine Ecology Progress Series*, **409**, 131–142.

GRIZEL H. (1985). Etudes des récentes épizooties de l'huître plate *Ostrea edulis* L. et de leur impact sur l'ostréiculture bretonne. Thèse de doctorat, Université des Sciences et Techniques de Languedoc, Montpellier, France

HERVIO D., BACHERE E., BOULO V., COCHENNEC N., VUILLEMIN V., LE COGUIC Y., CAILLETAUX G., MAZURIE J. & MIALHE E. (1995). Establishment of an experimental infection protocol for the flat oyster *Ostrea edulis* with the intrahaemocytic protozoan parasite *Bonamia ostreae*: application in the selection of parasite-resistant oyster. *Aquaculture*, **132**, 183–194.

HINE P.M., COCHENNEC-LAUREAU N. & BERTHE F.C.J. (2001). *Bonamia exitiosus* n. sp. (*Haplosporidia*) infecting flat oysters *Ostrea chilensis* (Philippi) in New Zealand. *Dis. Aquat. Org.*, **47**, 63–72.

LOPEZ-FLORES I., SUAREZ-SANTIAGO V.N., LONGET D., SAULNIER D., CHOLLET B. & ARZUL I. (2007). Characterization of acting genes in *Bonamia ostreae* and their application to phylogeny of the *Haplosporidia*. *Parasitology*, **134**, 1941–1948.

LYNCH S.A., ARMITAGE D.V., COUGHLAN J., MULCAHY M.F. & CULLOTY S.C. (2006). Investigating the possible role of benthic macroinvertebrates and zooplakton in the life cycle of the haplosporidian *Bonamia ostreae*. *Exp. Parasitol.*, **115 (4)**, 359–368.

LYNCH S.A., ARMITAGE D.V., WYLDE S., MULCAHY M.F. & CULLOTY S.C. (2005). The susceptibility of young prespawning oysters, *Ostrea edulis*, to *Bonamia ostreae*. *J. Shellfish Res.*, **24**, 1019–1025.

LYNCH S.A., ABOLLO E., RAMILLO A., CAO A., CULLOTY S.C. & VILLALBA A. (2010). Observations raise the question if the Pacific oyster, *Crassostrea gigas*, can act as either a carrier or a reservoir for *Bonamia ostreae* or *Bonamia exitiosa*. *Parasitol.*, **137**, 1515–1526.

MARTY G., BOWER S., CLARKE K., MEYER G., LOWE G., OSBORN A., CHOW E., HANNAH H., BYRNE S., SOJONKY K. & ROBINSON J. (2006). Histopathology and a real-time PCR assay for detection of *Bonamia ostreae* in *Ostrea edulis* cultured in western Canada. *Aquaculture*, **261**, 33–42.

MIALHE E., BOULO V., ELSTON R., HILL B., HINE M., MONTES J., VAN BANNING P. & GRIZEL H. (1988). Serological analysis *Bonamia* in *Ostrea edulis* and *Tiostrea lutaria* using polyclonal and monoclonal antibodies. *Aquat. Living Resour.*, **1**, 67–69.

MONTES J., ANADON R. & AZEVEDO C. (1994). A possible life cycle for *Bonamia ostreae* on the basis of electron microscopy studies. *J. Invertebr. Pathol.*, **63**, 1–6.

NACIRI-GRAVEN Y., MARTIN A.G., BAUD J.P., RENAULT T. & GERARD A. (1998). Selecting the flat oyster *Ostrea edulis* (L.) for survival when infected with the parasite *Bonamia ostreae*. *J. Exp. Mar. Biol. Ecol.*, **224**, 91–107.

PICHOT Y., COMPS M., TIGE G., GRIZEL H. & RABOUIN M.A. (1979). Recherches sur *Bonamia ostreae* gen. n., sp. n., parasite nouveau de l'huître plate *Ostrea edulis* L. *Rev. Trav. Inst. Pêches Marit.*, **43**, 131–140.

ROBERT M., GARCIA C., CHOLLET B., LOPEZ-FLORES I., FERRAND S., FRANÇOIS C., JOLY J.-P. & ARZUL I. (2009). Molecular detection and quantification of the protozoan *Bonamia ostreae* in the flat oyster, *Ostrea edulis*. *Molecular Cellular Probes*, **23**, 264–271.

VAN BANNING P. (1990). The life cycle of the oyster pathogen *Bonamia ostreae* with a presumptive phase in the ovarian tissue of the European flat oyster, *Ostrea edulis*. *Aquaculture*, **84**, 189–192.

*
* *

NB: There is an OIE Reference Laboratory for Infection with *Bonamia ostreae*
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on Infection with *Bonamia ostreae*