5.2.7 Bonamiasis of Oysters

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A. Name of Disease and Etiological Agent

Bonamiasis, or hemocytic parasitosis, is a disease of oysters caused by members of the genus *Bonamia* (Pichot et al. 1980). Molecular studies (Carnegie et al. 2000, Reece et al. 2004) confirmed ultrastructural evidence that these protistan parasites belong in the Haplosporidia despite the absence of a spore-forming stage in the life cycle. *Bonamia* spp., together with *Mikrocytos roughleyi and M. mackini* were historically termed 'microcell' disease agents due to their small size (2-3µm). There are two described species, *Bonamia ostreae* and *B. exitiosa* (formerly *B. exitiosus*, Berthe and Hine, 2003). Solid genetic evidence indicates that *Mikrocytos roughleyi* should also be placed in the genus *Bonamia* (Cochennec-Laureau et al. 2003b, Carnegie and Cochennec-Laureau 2004, Reece et al. 2004).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

a. *Bonamia ostreae*
Present in Western Europe from Mediterranean and Atlantic Spain northward to Denmark, including England and Ireland. Also present in British Columbia, Canada and California, Maine and Washington, U.S.A.

b. *Bonamia exitiosa*
Present in New Zealand.

c. *Bonamia* with uncertain species designation. Parasites that clearly belong in the genus *Bonamia* but have not been assigned to either described species or as new species have been reported in Australia, Chile and North Carolina, U.S.A (Burreson et al. 2004). *Mikrocytos roughleyi* which is described from Australia also appears to belong in this genus.

2. Host Species

a. *B. ostreae*
Found in cultured and wild European flat oyster *Ostrea edulis* populations in Europe and cultured *O. edulis* in the United States and British Columbia, Canada. The Suminoe oyster *Crassostrea ariakensis* [= *rivularis*] and the Southern Hemisphere flat oysters *Ostrea chilensis*,

O. puelchana, and *O. angasi* are also susceptible to infection. Attempts to establish infections did not succeed for the Pacific oyster *Crassostrea gigas,* the Manila clam *Venerupis [= Ruditapes] philippinarum,* the carpet-shell clam *Ruditapes decussatus,* the European cockle *Cerastoderma edule* and the mussels *Mytilus edulis* and *M. galloprovincialis*.

b. *B. exitiosa*
Found in *Ostrea chilensis* in New Zealand.

C. Epizootiology

Characteristics are generally similar for *Bonamia ostreae* and *B. exitiosa*. Transmission is direct and can be achieved by injection of infected tissue, by laboratory cohabitation with infected oysters or by deployment in endemic locations. Oysters begin to die 4-6 months after exposure and mortality can reach 80% or more in naïve host oyster populations, while those with a multi-year exposure history experience 20-60% mortality each year. Mortality in cultured oysters may be reduced by practices such as off-bottom culture. Prevalence and intensity increase during warm water periods. Prevalence of *B. ostreae* is much higher in *O. edulis* two years or older compared to younger oysters. Populations of *O. edulis* with a degree of resistance to *B. ostreae* infection have developed in Washington, U.S.A. (Elston et al. 1987), Ireland (Culloty et al. 2001), and elsewhere in Europe (Culloty et al. 2004), and have been experimentally selected for in France (Naciri-Graven et al. 1998). Bonamiasis was apparently transplanted from California, U. S. A. to France (Elston et al. 1986), Spain (Cigarría and Elston, 1997), Washington, U.S. A. (Elston et al. 1986) and Maine, U. S. A. (Friedman and Perkins 1994) via the importation of *B. ostreae*-infected *O. edulis*. Infection of New Zealand *Ostrea chilensis* with *Bonamia exitiosa* is enhanced by the presence of an undescribed apicomplexan parasite (Hine, 2002).

D. Disease Signs

Most oysters infected with *B. ostreae* or *B. exitiosa* have a normal appearance. Gill ulceration may be present in advanced cases (Bucke and Feist, 1985, Dinamani et al. 1987). The key disease feature is the presence of one, several or more 2-3 µm cells in the cytoplasm of granular hemocytes wherever they occur in the open circulatory system, such as in connective tissue of the mantle, heart, viscera or gills. Focal aggregations of infected hemocytes are often associated with intense infiltration of uninfected hemocytes. *B. exitiosa* may be abundant in the gonad (Dinamani et al. 1987, Hine, 1991, Hine et al. 2001).

E. Disease Diagnostic Procedures

1. Screening and Presumptive Diagnosis

Screening and presumptive diagnosis are accomplished by examining heart imprints, histological sections or hemolymph preparations as described below (Section E.2. and Table 1). A polymerase chain reaction protocol (Section F.1.) may also be useful for screening.

A fluorescent *in situ* hybridization method (Carnegie et al. 2003) using DNA probes based on the *B. ostreae* small subunit (18S) rDNA gene sequence may support the conclusion of a histological diagnosis. The probes hybridized with *B. ostreae* and not with the related protist *Haplosporidium nelsoni* although other protists (including *B. exitiosa*) were not examined. Positive hybridization results should not be interpreted as genus- or species-level confirmation until specificity is further defined.

A separate *B. ostreae* chromogenic *in situ* hybridization method using different DNA probes (also based on the small subunit rDNA gene sequence) has been described (Cochennec et al. 2000, Diggles et al. 2003). These probes hybridized with *B. exitiosa*, *H. nelsoni* and another unrelated Haplosporidian as well as with *B. ostreae*, but did not hybridize with *Mikrocytos mackini*. This protocol is not recommended because it is not genus-specific.

Presumptive differential diagnosis of *B. ostreae* (Europe, British Columbia, Canada and California, Maine and Washington, U.S.A) and *B. exitiosa* (New Zealand) can be made based on geographic location since the two species have not been reported to co-occur at these locations. Presumptive differentiation of *B. exitiosa* from *M. roughleyi* can be made on the basis of host species (*B. exitiosa* in *Ostrea chilensis*; *M. roughleyi* in *Saccostrea glomerulata*). The PCR product from a protocol that amplifies DNA of *B. ostreae*, *B. exitiosa* and *Mikrocytos roughleyi* can be used in a restriction fragment length polymorphism analysis to distinguish the species (Section F.1.b and Table 1 below; Cochennec-Laureau et al. 2003b; Carnegie and Cochennec-Laureau 2004).

2. Confirmatory Diagnosis

Histological preparations, stained heart ventricle imprints or stained hemolymph preparations can be used (Table 1). Histology may have slightly lower sensitivity (Diggles et al. 2003, Mirella da Silva and Villalba, 2004). Definitive differential diagnosis of *B. ostreae* and *B. exitiosa* requires transmission electron microscopy (E.2.c below) preferably in conjunction with restriction fragment length polymorphism analysis (F.1.b below).

a. Heart ventricle imprint or hemolymph preparation

Excise heart ventricle and make impressions on a standard microscope slide. Air dry, fix in methanol and stain with Giemsa, Wright-Giemsa or an equivalent stain (e.g., Hemacolor, Merck). The organisms are present in the cytoplasm of granular hemocytes and may be larger (2-5µm) than in histological preparations. Hemolymph drawn from the adductor muscle or pericardial cavity may also be used, and cytocentrifugation, if available, may improve visualization and sensitivity (Mirella da Silva and Villalba, 2004). The heart imprint method can be used with tissues from previously frozen oysters (Rogan et al. 1991). A representative heart imprint is shown in Figure 1.

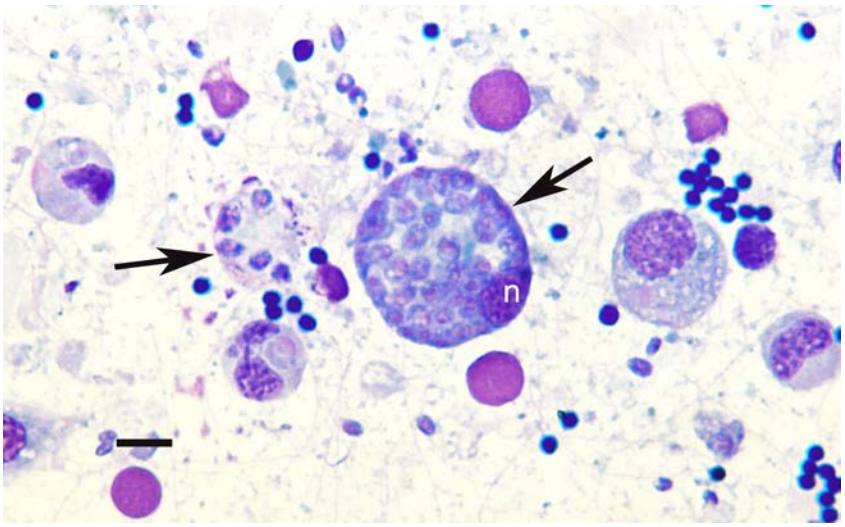

Figure 1. *Ostrea edulis* heart ventricle imprint, showing infection with *Bonamia ostreae.* The arrow on the right points to an intact hemocyte (with nucleus, n) filled with the parasites. The left arrow points to a burst hemocyte containing several of the parasites. The small, intensely blue spheres scattered throughout the preparation are heads of sperm. Scale bar = 10 µm, micrograph courtesy of Ralph Elston.

b. Histology

Routine histological procedures are used. Place 5 mm thick sections of oyster containing gill, cardiac cavity, visceral mass and mantle in a fixative such as Davidson's solution for 24 hours. Stain 5-6µm deparaffinized tissue sections with Harris' hematoxylin and eosin. With *Bonamia* spp. infection, 2-3µm cells are observed within hemocyte cytoplasm at 600-1000x magnification (Figure 2). Infected hemocytes may be present throughout the circulatory system, particularly in vascular sinuses of the stomach, intestine and digestive gland. Severe infections are often associated with massive inflammation that aids in diagnosis (Balouet et al. 1983, Bucke and Feist 1985, Cochennec-Laureau et al. 2003a). The parasites may be present free within the hemolymph or, based on one report, within gill epithelial cells (Montes et al. 1994). Hemocytes infected with *B. exitiosa* may be abundant in the gonad, particularly in males (Hine 1991).

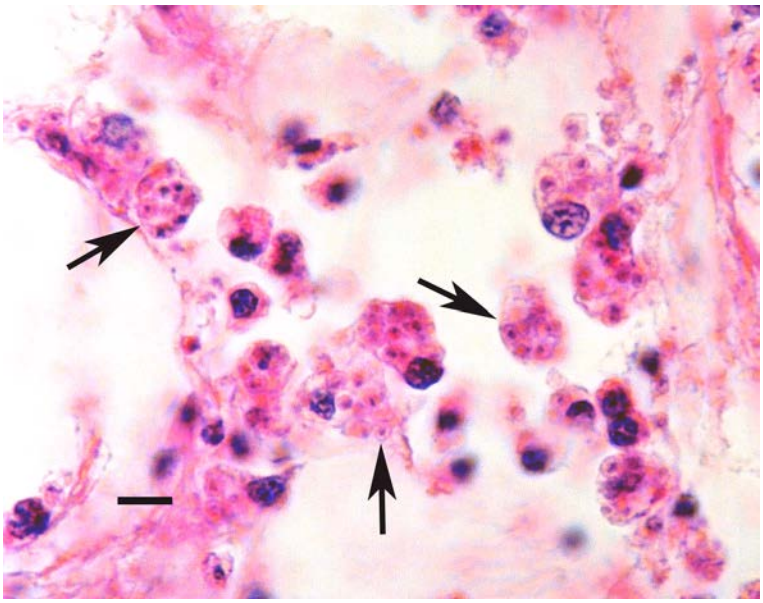

Figure 2. Histological preparation of *Ostrea edulis* infected with *Bonamia ostreae.* Arrows point to infected hemocytes. Hematoxylin and eosin. Scale bar = 10 µm, micrograph courtesy of Ralph Elston.

c. Transmission Electron Microscopy

Fix 1mm cubes of heart ventricle in 2.5% glutaraldehyde in 0.2µm filtered seawater for 2 hours, wash twice in filtered seawater, post-fix in 1% OsO_4 in filtered seawater, stain with 5 % uranyl acetate in 0.1M sodium acetate buffer for 45 min., dehydrate through an ascending series of 50-100% ethanol and embed in Embed It™ Low Viscosity Epoxy Kit (Polysciences). Stain sections in 5 % uranyl acetate for 10 min and 5 % lead citrate for 6 min (Hine et al. 2001). The parasites are present in parasitophorous vacuoles in granular hemocytes throughout the circulatory system. Key features include the presence of haplosporosomes, large mitochondria with sparse cristae, a relatively large nucleus with prominent, peripheral nucleolus and intact nuclear membrane during division. The two species differ in mean size and mean number of haplosporosomes, lipid droplets and mitochondria (Balouet et al. 1983, Dinamani et al. 1987, Hine et al. 2001).

F. Procedures for Detecting Subclinical Infections

1. Polymerase Chain Reaction

A PCR protocol based on 18S-ITS rDNA sequence has been developed for detection of *B. ostreae* (Protocol C, Carnegie et al. 2000). It provides an efficient and apparently sensitive method for screening large numbers of oysters, although specificity remains undefined. A separate PCR protocol based on the SSU rDNA sequence of *B. ostreae* was reported by Cochennec et al. 2000. This protocol also amplifies DNA of *B. exitiosa* (Diggles et al. 2003) and *Mikrocytos roughleyi* (Cochennec-Laureau et al. 2003b) although RFLP analysis provides distinction between the three species.

a. PCR to detect *Bonamia ostreae* (adapted from Carnegie et al. 2000, Protocol C).
Remove the right or upper valve and excise 0.1 gm gill tissue or draw 200 µl hemolymph from the pericardial cavity with a 22g needle on a sterile syringe. Extract and purify DNA using a QIAamp DNA Mini Kit (Qiagen, Inc.) following the manufacturer's 'Tissue Protocol' for gill tissue and 'Blood and Body Fluid Spin Protocol' for hemolymph respectively.
Forward primer C_F: CGGGGGCATAATTCAGGAAC
Reverse primer C_R: CCATCTGCTGGAGACACAG
Prepare 25 µl reaction mixtures containing 23 µl of master mix (1x PCR buffer [Invitrogen], 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.25 µM of forward and reverse primers, 0.6 U Platinum® Taq DNA polymerase [Invitrogen]) and 2 µl of template DNA at a concentration of 100 ng/µl. Follow an initial denaturation at 94 ºC for 4 minutes with 35 reaction cycles (94 °C for 1 min, 59 ºC for 1 min, 72 ºC for 1 min) and a 10 min extension at 72 ºC. Visualize the 760 base-pair amplicon using agarose gel electrophoresis (Chapter 6).

b. PCR to detect *B. ostreae* or *B. exitiosa* (adapted from Cochennec et al. 2000).
Extract DNA from gill or hemolymph using the procedure described above.
Forward primer BO: CATTTAATTGGTCGGGCCGC
Reverse primer: BOAS: CTGATCGTCTTCGATCCCCC
Prepare 50 µl reaction mixtures containing 45 µl of master mix (1x PCR buffer [Promega], 2.5 mM $MgCl_2$, 0.2mM dNTP mix, 1.0 µM of forward and reverse primers, and 1 U Taq DNA polymerase [Promega]) and 100 ng template DNA in a volume of 5 µl. Denature for 5 min at 94 ºC, then follow 30 reaction cycles (94 °C for 1 min, 55 ºC for 1 min, 72 ºC for 1 min) with a 10 min extension at 72 ºC. Visualize amplicons (300 bp for *B. ostreae*, 304 bp for *B. exitiosa*) using agarose gel electrophoresis (Chapter 6). A PCR-restriction fragment length polymorphism (RFLP) assay on the resulting product can be used to differentiate *B. ostreae*, *B. exitiosa* and *M. roughleyi* (Carnegie and Cochennec-Laureau 2004). In individual 200 µl tubes, add 16.3 µl of sterile distilled water, 2 µl of (10X) buffer provided with each enzyme, 0.2 µl of acetylated BSA (10.0 µg/µl, provided with enzyme) and 1.0 µl of BO-BOAS PCR product. Mix by pipetting, then add 0.5 µL of the restriction enzyme Hae II or Bgl I (Promega). Incubate at 37 ºC for 4 hours and visualize by electrophoresis in 2 % agarose gels. The 300 bp *B. ostreae* product is digested by both Hae II (producing 115- and 189-bp fragments) and Bgl I (producing 120- and 180-bp fragments). The 304-bp *B. exitiosa* product is digested by Hae II (producing 115- and 189-bp fragments) but not by Bgl I. The 304-bp *M. roughleyi* product is not digested by either enzyme. A 25-bp ladder (Invitrogen) will assist in size estimation.

2. *In situ* Hybridization

In situ hybridization for *B. ostreae* (adapted from Carnegie et al. 2003). Reaction with *B. exitiosa* is undefined.
Order oligonucleotide probes labeled with 5' fluorescein isothiocyanate (FITC) and purified by HPLC (Invitrogen).

Probe cocktail:
UME-BO-1: CGAGGCAGGGTTTGT
UME-BO-2: GGGTCAAACTCGTTGAAC
UME-BO-3: CGCTCTTATCCACCTAAT

Reaction positive control probe, binds oyster tissue and *Bonamia* spp:
UME-OE-385: TCATGCTCCCTCTCCGG

The following treatments should be included:

1. Diagnostic sample treated with the probe cocktail. *Bonamia* spp should be labeled if present.
2. Diagnostic sample treated with reaction positive control probe. This demonstrates probe binding and the ability to detect fluorescence signals when present.
3. Diagnostic sample with no probe (prehybridization buffer only). This is essential to determine the degree and nature of tissue autofluorescence.
4. Known *Bonamia*-positive sample treated with probe cocktail.
5. Known *Bonamia*-negative sample treated with probe cocktail.

Fix 5 mm transverse sections of oyster tissue, including gill, mantle and digestive gland in Davidson's solution for 24 hr. Place 5 µm sections on aminoalkylsilane-treated slides (Silane-Prep, Sigma). Deparaffinize in xylene or a xylene substitute (10 min, 3x) and rehydrate through 100 %, 95 %, 80 % and 70 % ethanol for 10 min each followed by PBS (Sigma) for 10 min and a separate volume of PBS for 5 min. Digest in Proteinase K (Sigma P4850 diluted to 3.0U/ml in PBS, 15 min, 37 °C) and wash in PBS with 0.2 % glycine for 5 min. Acetylate in a freshly made solution of 5 % (v/v) acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min at room temperature. Wash in PBS for 10 min and place in 5 x SET (750 mM NaCl, 6.4 mM EDTA, 100 mM tris base) for 10 min. Drain excess SET and place prehybridization buffer (5 x SET with 0.02% bovine serum albumin and 0.025 % sodium dodecyl sulfate) on each tissue section, incubate 30 min at 45 °C. Replace with prehybridization buffer containing the oligonucleotide probe or oligonucleotide cocktail. For the oligonucleotide cocktail set the highest probe concentration to 12 ng/µl. Coverslip with Parafilm and incubate in a humid chamber overnight at 45 °C. Wash in 0.2 x SET (25-fold dilution of 5x SET; 5min, 3x, 45 °C), and coverslip using Vectashield mounting medium (Vector Technologies). View under an epifluorescence microscope with a filter set appropriate for viewing FITC (absorbance = 490-496 nm, emission = 514-521 nm).

2. Immunological

ELISA (Cochennec et al. 1992) and IFAT (Boulo et al. 1989) techniques using a monoclonal antibody that binds the parasite membrane have been described for detection of *B. ostreae*. These methods do not appear to be widely used since the advent of molecular methods. Antigenic differences may exist between geographic isolates (Zabaleta and Barber, 1996).

Table 1. Methods for screening, presumptive diagnosis and confirmatory diagnosis of *Bonamia* spp. infection. Within the table, (-) indicates that the method is unsuitable due to cost or accuracy; (+) indicates the method has merit in some situations; (++) indicates a useful method with adequate sensitivity and specificity; (+++) indicates a recommended method on the bases of efficiency, cost, sensitivity and specificity. Consideration of the geographic location and host species will play a significant role in diagnostic interpretations and choice of diagnostic method.

Method	Screening	Presumptive	Confirmatory
Stained heart imprint or hemolymph preparation	+++	+++	+++
Histopathology	+++	+++	+++
Transmission Electron Microscopy	-	+	+
PCR for *B. ostreae*[1]	+++	+	-
PCR for *B. ostreae* or *B. exitiosa*[2]	++	-	-
In situ hybridization for *B. ostreae*[3]	-	++	-

1: Carnegie et al. 2000, Protocol C. Reactivity with *B. exitiosa* is undescribed.
2: Cochennec et al. 2000, Diggles et al. 2003.
3: Carnegie et al. 2003. Reactivity with *B. exitiosa* is undescribed.

G. Procedures for Transportation and Storage of Samples

Whole, live oysters should be kept cold (with gel ice packs) and moist (with towels or foam soaked in seawater) during transportation, and should be processed within 24-48 hours. Gaping, moribund oysters should be kept cold and processed as soon as possible. Air-dried heart imprints can be stored indefinitely at room temperature before staining. If only frozen samples are available, PCR screening should be used along with preparation of imprints from thawed heart tissue.

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