Bonamiasis in Australian *Ostrea angasi*

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Part 1. Diagnostic Overviews for Important Aquatic Animal Diseases.

Summary

Bonamiasis is a parasitic disease of flat oyster, which has resulted in catastrophic mortalities and decimated the flat oyster industries in several countries. In its *Aquatic Animal Health Code*, the OIE lists two *Bonamia* species, differentiated on ultrastructural and sequence differences: *Bonamia ostreae* in several northern hemisphere locations, and *B exitiosa* as described in *Ostrea chilensis* from New Zealand. These are characterised by similar lesions reflecting systemic haemocyte infection with the parasite. *Bonamia* sp parasites of less certain affinity, and associated with lower mortality, are known from other locations, including Australia. Sequence data from one Australian strain suggests it is similar but not identical to *B exitiosa* from New Zealand. The lesions also differ from those described for *Bonamia* species described in the OIE *Manual of Diagnostic Tests for Aquatic Animals*.

Diagnosis. In highly susceptible populations, such as those in Europe and New Zealand, bonamiasis is seen as a lethal infection of the haemocytes of flat oysters. Infection with these intrahaemocytic protozoans quickly becomes systemic in susceptible hosts, with overwhelming numbers of parasites coinciding with the death of the oysters. In Australian oysters, *Bonamia* parasites are usually found in or near epithelia, apparently restricted to these sites by a marked host haemocyte response, and rarely become systemic, resulting in limited mortality and parasite multiplication. The parasites may be present in very low numbers and be difficult to detect microscopically in infected oysters. For this reason, methods recommended by the OIE that are based on detection of systemic spread may not be applicable to Australian stocks. Clinical signs are limited and relatively non-specific. Diagnosis of bonamiasis is therefore based on a range of procedures. Presumptive diagnosis is made on histopathological observations. Electron microscopy may be used to confirm the identity of the parasite, when abundant. Tests to detect the presence of the parasite when numbers may be low include real-time Taqman polymerase chain reaction (PCR) and conventional PCR. Sequencing of PCR products is performed to partially differentiate the strain of the parasite. In addition, sequencing is necessary because amplicons of similar sizes are obtained from non-infected and *Bonamia*-infected oyster tissues. In that respect, the restriction fragment length polymorphism (RFLP) assay becomes redundant and will not be included in this document.

Introduction

Bonamia ostreae was first described as the causative agent of substantial mortalities of the European flat oyster, *Ostrea edulis*, in France in 1979.[1,2] Later evidence indicated the parasite had been introduced to the naïve and susceptible population with oyster movements.[3] Bonamiasis with significant mortality caused by this pathogen is also found in oyster farming areas of Spain, the Netherlands, Ireland and southern parts of the United Kingdom[4,5,6,7,8] and in the USA.[3,9,10] Not all infected populations of *O edulis* in the USA show high mortality.[9] High mortalities associated with another species of the genus *Bonamia*, *B exitiosa*, was reported in the New Zealand dredge oyster *Ostrea chilensis*.[11] *Bonamia* parasites of uncertain affinity, associated with lower mortality, have been reported in the Australian flat oyster, *Ostrea angasi*[12,13], and in Argentina[14], Chile[15], and the USA.[16,17] Other recognised *Bonamia* species include *Bonamia (*formerly *Mikrocytos) roughleyi,* the parasite associated with the disease winter mortality in Sydney rock oysters, *Saccostrea commercialis,* and *Bonamia perspora* of *Ostreola equestris,* the only known spore-producing *Bonamia* species.[18]

The mortality associated with *Bonamia* parasites in Australian flat oysters is variable[19,20,21] and, apart from any parasite factors, may reflect genetic differences in population resistance as well as stress factors. From an epidemiological perspective, it is of utmost importance to determine the relationship between the causative agents of these multiple microcell infections. The sustainability of oyster farming depends on good biosecurity and disease surveillance. The management of these pathogens and prevention of transfer of infected stocks depend on highly sensitive and specific diagnostic methods. Detection during routine broad-scale histological disease surveillance is limited by poor sensitivity to the low levels of infection that may be present, but an appreciation of the pathology will identify suspect populations where

more sensitive and specific tests are required. Where a changed pattern of infection is seen, verification of the species and strain may also be important.

This ANZSDP covers methods and criteria for diagnosis of *Bonamia* sp in *O angasi* in Australia for both clinical and surveillance purposes. Methods for *B ostrea* and for *B exitiosa* in New Zealand are described by OIE.[22] The development of molecular methods allows the detection and differentiation of *Bonamia* species in oysters or in other host species and will be valuable diagnostic tools for use in life cycle studies.

Aetiology

Bonamia parasites are small (2-5 μm) thin-walled parasites frequently surviving within host haemocytes, but also seen in extracellular locations. They are protistans that belong to the Phylum Haplosporidia, within the Supergroup Alveolata, which comprises haplosporidians, dinoflagellates, ciliates and apicomplexans.[23,24] Recognition of *B ostreae* and *B exitiosa* as two separate species is based on ultrastructural differences in certain key organelles, existing between the two organisms, and differences in 18S and ITS sequences of rDNA, as well as discrepancies in the SSUrRNA sequences shown by PCR-RFLP.[25,26,27] Sequence data alignment with homologous genes of three *Bonamia* isolates showed one Australian (NSW) isolate to be closely related to the New Zealand isolate with only four base pair mismatches and eleven base pair deletions in the 18S rDNA gene sequence.[25] A close relationship to *Bonamia (Microcytos) roughleyi* was also demonstrated[41] and further research is needed, using multiple *Bonamia* sp isolates before the relationship between these *Bonamia* species is established.

Occurrence and Distribution of Bonamiasis in Australia

Bonamiasis has been detected in association with overt mortality in experimental stocks of *Ostrea angasi* in Victoria[19, 21] and in farmed stocks in Western Australia as a duel infection with herpes virus.[21, 28] Bonamiasis with little or no mortality has been seen in Tasmania, on some occasions in Western Australia, and more recently in NSW.[21,29] Surveys of *O angasi* in South Australia in 1992 and 1993 did not detect any *Bonamia* in that State.[21] *Bonamia*-like parasites were reported in the Pacific oyster, *Crassostrea gigas* in South Australia in 2003 [41], and it is suspected also in *O angasi* at that time.

Epidemiology

Unlike other Haplosporidia, the life cycle of typical *Bonamia ostreae* is direct, as bonamiasis can be transmitted experimentally by cohabitation or inoculation[30] and no secondary host is known or suspected. Spores have been detected in only one *Bonamia* species, *B perspora* from North Carolina, USA.[18] The prepatent period determined for *B ostreae* is up to 5 months, but there is not direct information from Australia and it appears that *Bonamia* sp infections may never reach clinical significance in some *O angasi* populations.

In general, infection persists in infected natural populations, or farmed populations not amenable to biosecurity measures, though the level of infection may vary between years. *Bonamia* spp are believed to be present in infected populations throughout the year, but prevalence and intensity of infection tend to increase during the warm

season.[7,20,31,32,33] Southern hemisphere infections shows the highest prevalences from January to April. In September and October, the parasite is undetectable following light infections, or barely detectable in populations that experienced significant recent mortalities.[31,32,20]

Both prevalence and severity in a population may depend on population density.[20] In Australian stocks, the presence of focal epithelial haemocyte infiltrations may be the only indications of infection in unstressed small populations, with the parasite undetectable in a single section through many of these lesions.

Clinical Signs

In Australian populations undergoing mortality (Victoria, 1991), gross signs of *Bonamia* sp infection included visibly poor condition, thin watery meats, heart enlargement, mantle recession, pale digestive gland and occasionally visible abscesses.[17,20] Similar signs have been observed in *O edulis* infected with *B ostreae*.[34, 35] *Bonamia ostreae* may cause yellow discoloration and extensive lesions on the gills and mantle of *O edulis*[1], but such lesions are extremely rare in *Ostrea chilensis* infected by *B exitiosa* in New Zealand. Gill lesions are also caused by *Bonamia* sp infecting *O angasi* in southern Australia, but are seldom visible grossly. Pale digestive systems, reflecting a reduction in feeding activity, are often the most common gross sign. However, gross signs of infection are rare in stable populations, and though some mortalities may occur, these may not involve loss of condition.[19,20] Even in susceptible *O angasi* populations, most of the infected oysters appear normal.[22]

Pathological Changes

Parasites of all *Bonamia* species may be seen within haemocytes or in extracellular locations. In *O angasi,* they are frequently seen within epithelia, but in this location they are also believed typically to be present within haemocytes intruded into the epithelium, rather than within epithelial cells (M Hine, pers com.). *Bonamia ostrea* parasites are able to block haemocyte killing mechanisms responses such as the production of toxic oxidative radicals mechanism[36,37] so in highly susceptible hosts the intrahaemocytic *Bonamia* parasites multiply and quickly become systemic. Parasite replication cycles stimulate a marked systemic haemocytosis, resulting in a loss of condition, with overwhelming numbers of parasites and circulating haemocytes coinciding with the death of the oysters. In contrast, haemocytes within the epithelium are often undergoing transmigration across the epithelium, resulting in removal of parasites to the gut lumen or external surfaces.

In oysters from Australia, *Bonamia* sp is usually found in or near epithelia, and rarely become systemic, apparently restricted to these sites by a marked host haemocyte response, resulting in limited parasite multiplication and few mortalites. The haemocytes appear more effective in killing the parasite as cells consistent with dead or dying bacteria may be common. Occasional similar findings have also been reported from *O chilensis* infected with *B exitiosa* in New Zealand.[32] The parasites may be present in very low numbers and be difficult to detect microscopically in infected oysters, even within the hallmark focal epithelial reactions that show haemocytes within and adjacent to affected epithelia.

Such focal reactions are most common in the digestive system, and the gills, but may also be found in the mantle and gonad. Occasionally the subsequent erosion of the gut or gonad epithelia results in massive accumulation of haemocytes and sloughed cells to give an abscess-like appearance, with very variable numbers of visible *Bonamia* sp parasites. The severity of focal lesions in *O angasi* varies between populations, ranging from abscesses of the peri-gut or gonad region, in which variable but often large numbers of *Bonamia* organisms are present, to small focal infiltrates in the of the gut or gill, in which a few intra-epithelial *Bonamia* parasites may sometimes be seen.

In *O angasi,* heavy systemic infection with dispersed *Bonamia* sp-laden haemocytes in haemic sinuses and interstitial tissue is rare except in beds experiencing heavy mortality. Intense epithelial-associated focal lesions are generally also present in these populations, and usually within the same oysters.

Diffuse haemocytosis without evidence of systemic *Bonamia* sp infection in circulating haemocytes is more common in oysters with multi-focal lesions, and may occasionally be accompanied by light systemic infection in less affected populations.[20]

For comparison, *Bonamia exitiosa* infections in *O chilensis* in New Zealand are diffuse and systemic, not focal, and rarely involve epithelia. Light infections are usually confined to the connective tissue of the digestive gland, but as infection intensity increases, the gills and mantle become progressively more heavily infected. Heavy infections in the digestive gland result in dissociation and lysis of connective tissue cells, leaving ragged spaces. Peak infections in summer coincide with re-absorbtion of unspent ova, during which *Bonamia exitiosa* enters the gonad in haemocytes. The haemocytes phagocytose lipid-rich ovacyte cytoplasm, and this is used as an energy source by the parasite[38] resulting in rapid proliferation.[31]

The pathological changes shown by Australian *O angasi* populations, with predominantly focal lesions, relatively few parasites and little mortality or evidence of overt disease, are consistent with USA flat oyster populations showing adaptation to *Bonamia ostrea*.[9]

Differential Diagnosis

Differential diagnoses for very light infections, which may show little except focal epithelial infiltrates in which the parasites may be hard to locate, includes non-specific response such as epithelial damage from focal tubule blockages by lodged copepods[19] or possibly algal bloom damage though the latter may provoke a more generalised response.

Diagnostic tests & specimens

Tests available

The presumptive diagnostic methods used for infection by any *Bonamia* species are histopathology, and cytological examination (tissue imprints), though the latter is of limited value in Australian infections, which are likely to be focal with low numbers of parasites. Tissue imprints, using heart as a source of circulating haemocytes, may provide a screening method with populations that are undergoing significant mortality and in which disseminated infection is more likely. Finding parasites within haemocytes on examination of smears provides a reliable positive diagnosis, but negative findings must be confirmed by histopathology and/or PCR.

Transmission electron microscopy examination is used for confirmatory identification of the pathogen. Molecular diagnostic methods can be used for the detection (real-time and conventional PCR) as well as for its genus and strains identification[25].

1. Disease diagnosis

Histopathology

Bonamiasis is diagnosed by findings on light microscopy of the typical pathological changes and of the *Bonamia* parasite within susceptible cells, which are usually haemocytes.

Tissue imprints

Tissue imprints, specifically the examination of haemocytes using heart imprints, is recommended by the OIE for detection of *B ostrea* and *B exitiosa*, but are considered unreliable for Australian *Bonamia* infections as these are characterised by focal reactions and an uneven distribution rather than systemic haemocyte infections.

Confirmatory tests

Electron microscopy may be used to confirm the identity of the parasite where these are abundant. Tests to detect the presence of the parasite when numbers may be low include PCR (real-time and conventional) and identification of the strain by sequencing[25].

2. Surveillance

Histopathology is recommended for routine surveillance of Australian populations of *O angasi* for *Bonamia* infection, as this monitors the severity of infections and the presence of confounding diseases. Tissue smears are inappropriate as infected populations may show negligible systemic *Bonamia* infection in circulating haemocytes, and in general may show few parasites, which are unevenly distributed, and may be bound within epithelia and therefore less likely to be present in smears.

Sampling must be undertaken at appropriate times of the year (generally January to May). Parasites may be few and difficult to find in many lesions. The Australian *Bonamia* parasite also stains poorly and may be difficult to see in sections. Small intra-epithelial haemocyte foci, especially early in the summer, must be treated with suspicion, even when parasites are not seen during examination of one or two sections. Regardless of the timing of exposure, the disease may be not detectable by

the usual methods during the first few months of infection (estimates vary, but possibly up to 5 months).

3. Exclusion

Methods for exclusion are the detection of the parasite or characteristic lesions by histopathology at appropriate times of the year (usually on more than one occasion), with confirmation by PCR techniques where suspicious lesions are present, or by the use of PCR techniques alone. These techniques have been shown to be more sensitive than histopathology for detection of the parasite.[39, 44] The detection of the characteristic (but not pathognomonic) focal lesions within or near epithelia as (an indicator of likely infection) is also more sensitive than the microscopic detection of parasites.[20]

Tissues to be examined

Tissue samples suitable for examination by histopathology or PCR include digestive gland and gills.

Storage of samples

Fixatives for histopathology must be isotonic with seawater. The OIE[22] recommends fixatives such as Davidson's or Carson's fixative. However, 10% formalin in seawater may be used and has been found to provide better visualisation of the parasite, which may be difficult to see in *O angasi*, as well as better differentiation of haemocyte types.

For PCR testing, samples must be fixed in 95% ethanol to maintain integrity of the *Bonamia* DNA. Commercial NA stabilizers, which avoid the transport issues associated with ethanol, can be used as an alternative to ethanol.

Part 2. Test Methods for Bonamiasis

Histopathology

OIE recommendations for histological examination[22] are that a transversal 'steak' is cut dorso-ventrally through the soft tissues and then immediately placed in fixative. With smaller samples, the whole animal, removed from the shell, can be fixed with a fixative, such as Davidson's, to provide optimal parasite definition. Formalin-based fixatives such as 10% formalin seawater can also be used, and will provide better differentiation of haemocyte types.

Several nonspecific stains enable *Bonamia* to be seen. However, *Bonamia* sp parasites in *O angasi* stain relatively poorly, regardless of the fixative or stain used. They are generally seen best with haematoxylin–eosin stain. Overstaining of other cell types should be avoided.

Because of the propensity for focal epithelial infection, sections should include digestive gland, mantle and gills. Gonad sections are also recommended by OIE for *Bonamia* infections, though infection is this tissue is less frequent in *O angasi.*

In many Australian infections, the parasite may be restricted to intra-epithelial locations, including the gill but especially the gut and occasionally the mantle. Although most parasites are probably within haemocytes in this location, the OIE recommends that a positive diagnosis requires that the parasite be observed inside the haemocytes. This may be difficult to achieve, as it may be difficult to identify individual cells as haemocytes at the light microscope level when they are within the epithelium, especially if they are distorted by the presence of the parasites. A presumptive positive diagnosis may be made on the basis of detection of typical parasites of this size range (2-5 µm) within haemocytes or within epithelia with associated haemocyte response (Figures 1 and 2). Infection in gonads can generally be identified as within haemocytes in this location.

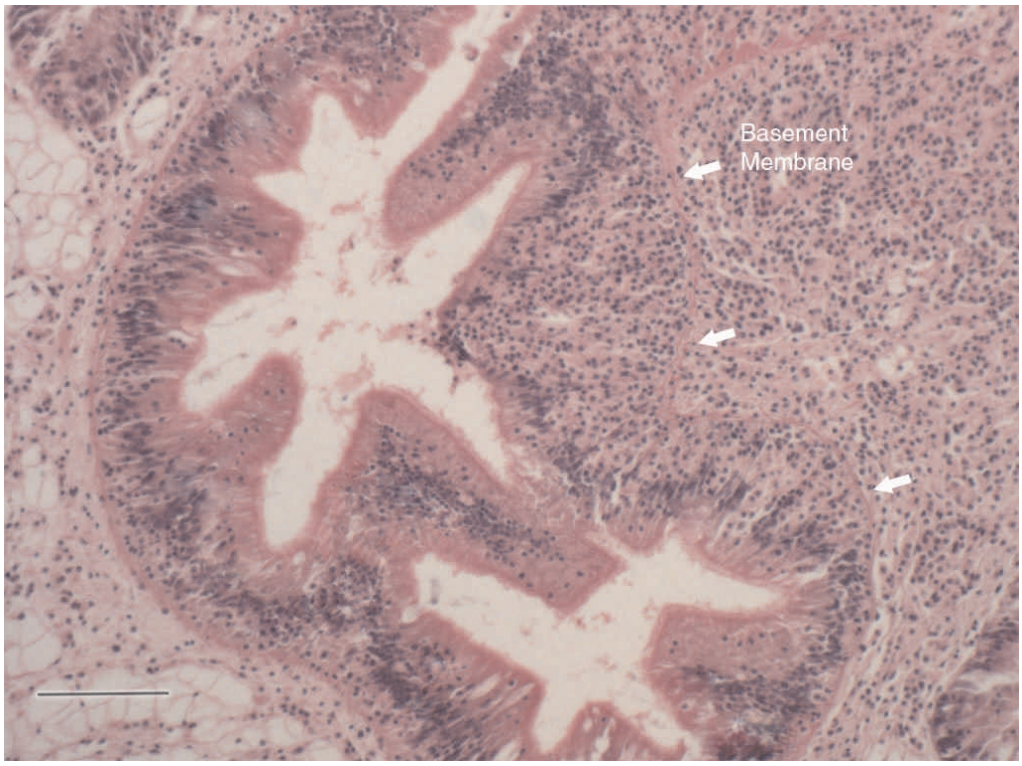

Figure 1. Infiltration of *O angasi* haemocytes into epithelium as a response to *Bonamia,* lifting the surface epithelium from the basement membrane (arrows). Haematoxylin and eosin (H&E) staining. Bar length 100 µm.

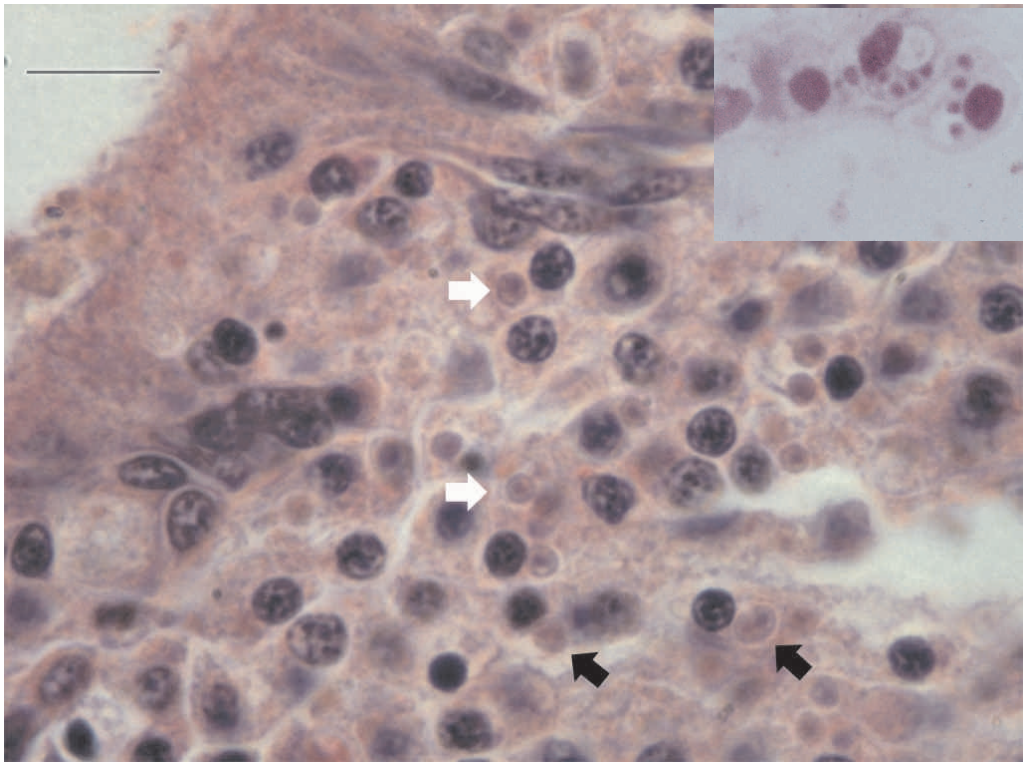

Figure 2. *Bonamia* parasites within haemocytes (arrows). Both single nucleate forms (white arrows) and replicating binucleate forms (black arrows) are present. H&E staining. Bar length 10 µm.

Insert: *Bonamia* parasites in haemocytes, heart smear stained with Hemocolor®, photographed under oil immersion.

Cytological examination: tissue imprints

Impression smears of oyster spat or heart tissue (ventricle preferably) are made on a glass slide. The heart is easy to find as a (partly) dark coloured organ located under the pericardium in the cardiac cavity between the visceral mass and adductor muscle. The heart is grasped with forceps excised with scissors, blotted onto blotting paper or paper towel, and dabbed rapidly and repeatedly along a slide multiple time to provide a series of impressions of different levels of moisture so that an optimum imprint can be found after staining.

The slides are air-dried and fixed in methanol. The prepared imprints are stained using a commercially available blood staining kit (Hemocolor® or Dif-Quick®), in accordance with the manufacturer's instructions. After staining, the slides are rinsed in tap water, allowed to dry completely in cold or warm air, mounted with a cover-slip using an appropriate synthetic resin and examined using oil immersion. Each slide needs to be examined for 10 minutes before being assessed as negative.

The parasite (2-5 µm in size) is seen inside or outside the haemocytes and has basophilic cytoplasm and an eosinophilic nucleus. Colours may vary with stain used. The organisms are spread and appear enlarged by this method compared with those observed in histology.

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Agent isolation

No method is available. Concentration techniques by density centrifugation are available to provide material for further study, but are not used for diagnosis.

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Agent identification by electron microscopy

The OIE recognises that differences exist between *Bonamia* sp forms and *Bonamia ostreae:* number of haplosporosomes and large globules, morphology of mitochondria, smaller nucleus and cytoplasm ratio.[1,11] Plasmodial forms of *Bonamia* 355 sp are distinguished by their size (4.0–4.5 µm), irregular cellular and nuclear outline, amorphous cytoplasmic inclusions (multi-vesicular bodies), and arrays of Golgi-like smooth endoplasmic reticulum. Forms of intermediate cytoplasmic density are more electron dense than the plasmodial forms and slightly smaller in diameter (3.0–3.5 µm). Haplosporosomes are formed from Golgi/nuclear material complexes and are 360 similar in construction and structure to some viruses.[40]

Detection by PCR assay (conventional and real-time TaqMan) and identification by sequencing

365 *Introduction*

As Bonamia cannot be propagated *in vitro,* molecular methods such as the PCR are particularly important for its diagnosis. PCR methods described here are genus specific only. Sequencing of the amplicons allows partial differentiation of strains and 370 may also allow a molecular epidemiological analysis, which may be important for the control, prevention and eradication of bonamiasis.

Real-time PCR provides a real-time monitoring of PCR amplification, and quantification of target nucleic acid in the sample. The ability to include internal controls in each reaction tube by multiplexing methods provides a convenient and 375 rapid way to confirm the quality of the sample DNA. The PCR reaction and analysis is conducted in a closed tube system, substantially reducing the risks of cross-contamination.

Procedures

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Sample preparation
Due to the sensitivity of PCR tests, care must be taken at every step of sample preparation to ensure that cross-contamination of diagnostic samples does not occur. Thus all instruments and sample containers must be clean and uncontaminated, i.e. not 385 previously exposed to aquatic animal pathogens.

Samples must be handled and processed using sterile disposable single-use containers, instruments and reagents to minimise the risks of contamination of the samples. As a

general principle, samples to be used in the PCR suite for molecular diagnosis will be inactivated by an approved method prior to movement to the PCR suite.

Inactivation will be carried out by the following procedures by staff approved to work with the categories of agents.

About 20 mg of tissues are cut from each sample using sterile scissors and tweezers and put in a labelled, sterile 1.5 mL vial (conical bottom). Tissues are mashed using a disposable plastic pestle, and 180 µL of ATL buffer (provided in the commercial QIAamp DNA® kit) added to each vial.

Nucleic acids are extracted from submitted samples in the Biological Safety Cabinet Class II, in the PCR suite.

Nucleic acid extraction

Nucleic acid (including *Bonamia* DNA) is obtained from tissue samples using the QIAamp DNA® kit following manufacturer's instructions. Any extraction method could be used, however this method was validated using this kit and comparative testing should be undertaken if reagents are changed.

Diagnostic Real Time PCR TaqMan assay[31]

The TaqMan technology was chosen for the development of a rapid, sensitive and reliable molecular diagnostic assay specific for *Bonamia* at the genus level. Other real time systems (e.g. Corbett or Roche) could be used, however this method was validated using only TaqMan and comparative testing should be undertaken if the real time system is changed.

An ABI Prism®7700 Sequence Detection System and software Sequence Detector version 1.9 (PE Applied Biosystems) is used for the analysis and storage of data. Primers and probe for the multiplex TaqMan assay were designed using Primer Express Software version 1.5 (PE Applied Biosystems). The *Bonamia* primers and probe were based on the ITS-1 of the rRNA operon, a relatively conserved genomic region among *Bonamia* isolates. Primer and probe sequences are as follows:

Forward primer (ITS-For): 5'-CCCTGCCCTTTGTACACACC-3'

Reverse primer (ITS-Rev): 5'-TCACAAAGCTTCTAAGAACGCG-3'

6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA), labelled probe (Bon ITS): 6FAM-TTAGGTGGATAAGAGCCGC-TAMRA

The ITS primers are used at a final concentration of 900 nM.
The 18S endogenous control primers 18S Forward Primer 5'- CGG CTA CCA CAT CCA AGG AA -3' and 18S Reverse Primer 5'- GCT GGA ATT ACC GCG GCT -3' (TaqMan® Ribosomal RNA Control Reagents cat. # 4308329 Applied-Biosystems), used to validate the DNA extraction procedure and the absence of PCR inhibition, are used at a final concentration of 113 nM. The ITS FAM probe and the 18S control VIC probe 18S Probe (5'- TGC TGG CAC CAG ACT TGC CCT C -3' TaqMan® Ribosomal RNA Control Reagents cat. # 4308329 Applied-Biosystems) are used at a final concentration of 250 nM and 31 nM, respectively. The reactions are carried out in a 96-well plate in a 25 µL reaction volume containing 12.5 µL Universal Master

Mix (PE Applied Biosystems). Volumes (2 µL) (~100ng per µL) of each DNA sample are added. The following thermal cycling conditions are used:

- 50°C for 2 min
- 95°C for 10 min
- 35 cycles of 95°C for 15 sec and 63.6°C for 1 min.

All samples are tested in triplicate to ensure the reproducibility of the results.

A sample is considered positive when the change in fluorescence (ΔR_n) of FAM or VIC, relative to that of ROX (internal reference signal), exceeds the set threshold values of 0.05 for FAM and 0.05 for VIC in the linear range of the amplification plots at a cycle threshold (C_T) value below 35. C_T is defined as the cycle at which a statistically significant increase in fluorescence output above background is detected. Figure 3 presents an example of amplification curves from positive and negative DNA samples. In the eventuality that only one or two of the triplicate wells are positive, the decision would depend on the Ct value. For example, in a test where one well is negative and the other two wells are clearly positive (low Ct values) and of similar value, then this would suggest a positive result.

If one well is clearly positive and the other two negative, this test would be called invalid and should be repeated.
If one or two wells are weakly positives (high Ct values) close to the limit of detection, then this could be declared an indeterminate result or a positive with a lower level of confidence.

Bonamia *genus-specific PCR*[22]

The PCR mixture for a single sample consists of the following reagents:

- 9.5 µL of deionised sterile water
- 12.5 µL of HotStar Taq Master mix
- 0.5 µL of the *Bonamia* 18S rDNA forward primer Bo: CATTTAATTGGTCGGGCCG (18 µM)
- 0.5 µL of the reverse primer Boas: CTGATCGTCTTCGATCCCCC (18 µM)
- 2 µL of extracted DNA (~100 ng per µL)

For multiple samples, the volumes are increased stoichiometrically. The mixture is incubated in an automatic thermal cycler (Perkin Elmer GeneAmp 2400) that is programmed for:

- one cycle at 94°C for 15 min (activation of the Hotstar Taq polymerase)
- 35 cycles at 94°C for 1 min (denaturation), 59°C for 1 min (annealing) and 72°C for 1 min (extension)
- one cycle at 72°C for 7 min (final extension)

Amplified DNA (size: 300 bp) is detected by agarose gel electrophoresis (Figure 4).

The 18S primers described in the 'Diagnostic Real Time PCR TaqMan assay' section can be used, under the same amplification protocol as the primers Bo and Boas, to validate the DNA extraction procedure and the absence of PCR inhibition.

Any Taq enzyme could be used, however this method was validated only using HotStar Taq and comparative testing should be undertaken if reagents are changed.

Interpretation

At the completion of the PCR, the 300bp amplicon is identified by agarose gel electrophoresis and removed using a scalpel blade. DNA extraction from the gel is performed using a Qiaquick Gel Extraction (Qiagen) according to the manufacturer's instructions.

DNA sequence is determined by using the PCR primers Bo and Boas as sequencing primers. Sequence identity and genotype are determined by a Blast search of the Genbank database to confirm a positive result of a test for *Bonamia* spp. Note that Haplosporidian found in marine molluscs such as abalone can present high level of sequence identity with *Bonamia* spp. Further testing using the more specific TaqMan assay provides confirmation of suspicious cases.

Note that the DNA extracted from uninfected oyster tissues also produce a non-specific amplicon of similar molecular size as the positive oyster tissue samples, therefore sequencing of the amplicon is necessary to confirm its nature.

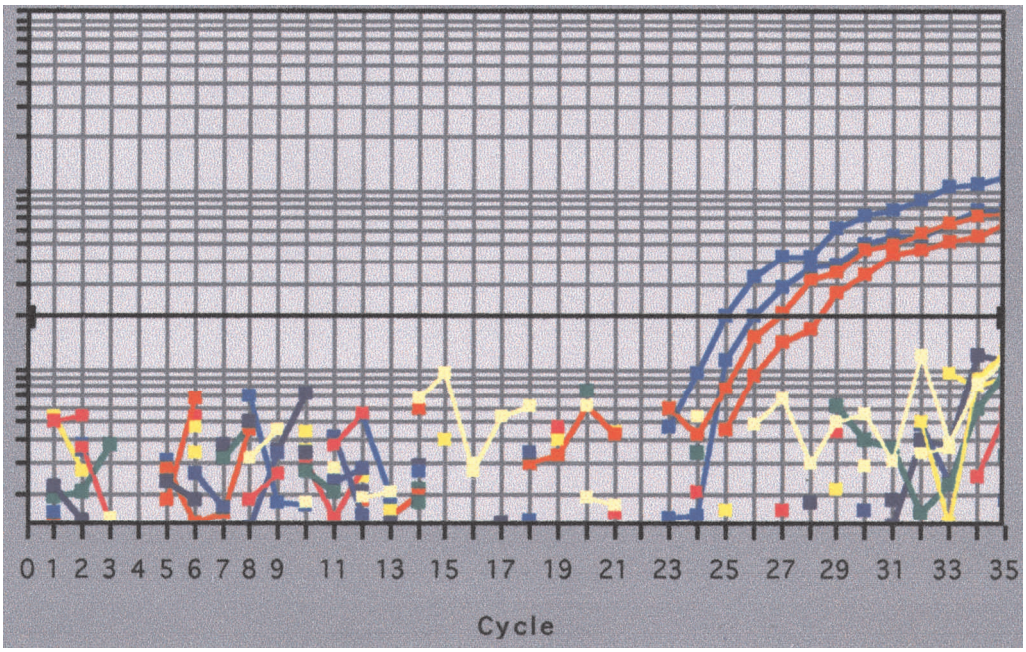

Figure 3. Real-time PCR amplification of *Bonamia* rDNA.

The graph shows the log of the change in fluorescence intensity (y-axis) versus the number of threshold cycles (C_t value) (x-axis) for each DNA sample. The difference in C_t value for each sample is indicative of different levels of *Bonamia* infection in oyster samples. Lines (e.g. yellow, green) that do not reach the threshold (black horizontal bar) represent negative control oyster samples. Note that in this case the TaqMan assay is performed in duplicate (there are two curves for each target concentration).

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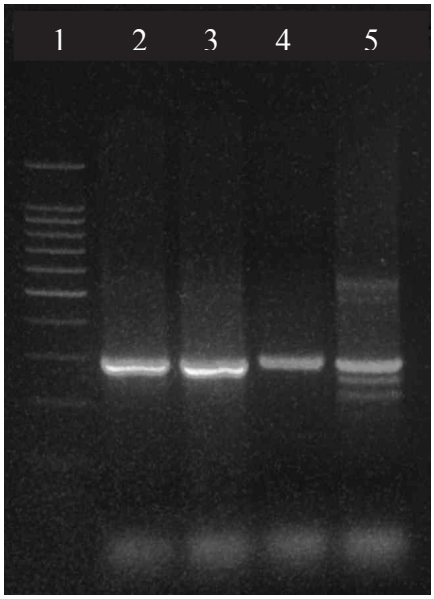

Figure 4. Conventional PCR Assay using the primers Bo-Boas for the identification of *Bonamia* DNA.

Photograph of an agarose gel containing ethidium bromide-stained, *Bonamia*-specific amplicon. The primers used for the amplifications were Bo and Boas producing a 300 bp amplicon. Lane 1 contains 100 bp ladder, lanes 2, 3 and 4 are samples of *Bonamia*-infected oyster tissues, and lane 5 contains uninfected control tissues. The strong band in lane 5, based on sequence, is oyster DNA. Because the uninfected oyster tissues present an amplicon of similar size as the infected tissues, sequencing is required for confirmation of positive samples.

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41. *Bonamia exitiosa* Technical Working Group Teleconference. Attendance: Jane Frances (Chair – NSW DPI), Rob Adlard (Qld Museum), Nicky Buller (WA

Fisheries), Serge Corbeil (AAHL), Mark Crane (AAHL), Marty Deveney (PIRSA), Jeffrey Go (NSW DPI), Judith Handlinger (Tas DPIW), Brian Jones (WA Fisheries), Ian Marsh (NSW DPI), Belinda Wright (DAFF). 22 May 2007.

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Part 3. Reagents and Kits for Important Aquatic Animal Diseases

Real-time PCR reagents

At AAHL, an ABI Prism®7700 Sequence Detection System and software Sequence Detector version 1.9 (PE Applied Biosystems) is used for the analysis and storage of data. Primers and probe for the multiplex TaqMan assay are designed using Primer Express Software version 1.5 (PE Applied Biosystems). Thus reagents used are those that are compatible with the ABI system.

Reagents stored at room temperature

QIAamp DNA Mini Kit (QIAGEN Cat # 51304 or equivalent) that includes all buffers and Proteinase K for DNA extraction.

Reagents stored at –20°C

Taq Master Mix (Applied Biosystems cat # 4318157 or equivalent)

Reagents stored at -70°C

18S endogenous control primers and VIC probe (Applied Biosystems cat # 4308329 or equivalent).

List of equipment for the TaqMan assay

Applied Biosystems 96 well Optical reaction plates

Applied Biosystems Adhesive cover kit or equivalent

Applied Biosystems Sequence detection system Model 7700

Macintosh Power Mac G4 computer and operating system

Applied Biosystems developed software SDS *v1.7*

Bench top centrifuge IEC model Centra and appropriate plate holders

Reagents for conventional PCR

Reagents stored at –20°C

Taq polymerase

dNTPs (1.25mM)

Mg^{2+}-free buffer 10X

$MgCl_2$ (25mM)

100% Ethanol AR grade

70% Ethanol

Primers (18μM)

HotstarTaq Mastermix

100bp DNA ladder & loading dye

Reagents stored at room temperature

QIAamp DNA Mini Kit (QIAGEN) that contains:

- DNA columns
- Buffer ATL
- Buffer AL
- Buffer AW1
- Buffer AW2
- Buffer AE
- Proteinase K

Agarose

Ethidium bromide

40 x TAE Buffer

List of equipment

Apart from the normal range of equipment required in the standard diagnostic laboratory (e.g. refrigerators, freezers, mixers, micropipettes, biological safety cabinets, centrifuges, balances, microwave oven, thermometers), specialised equipment required to undertake diagnostic PCR may include dry-heat blocks, thermocycler, gel electrophoresis equipment, UV transilluminator, camera system and nucleic acid sequencer.

Quality control

Molecular diagnosis should be operated under an ISO 17025 accredited and audited quality assurance program. Thus, such a program would include initial evaluation of kits and validation of performance; ongoing internal evaluation through mandatory use of appropriate quality control samples where available; and performance monitoring through quality assessment or proficiency programs.

External quality control samples over the appropriate range of testing must be obtained or manufactured wherever possible. Wherever possible, quality control samples should be included in every assay performed and the data presented so that run-to-run performance can be monitored. Positive, negative and reagent controls should be included as specified in the protocol. As a norm, formalin-fixed controls

would be included with formalin-fixed test samples and appropriate unfixed controls would be conducted with fresh tissue, culture supernatants or other test samples. Stocks of controls should be established. These controls should be evaluated prior to storage and used in a check-testing regimen and as controls for the conduct of disease investigations.

Nucleic Acid Storage Conditions

Positive control samples should be stored at/below –70^{o}C. Stocks and controls should be dispensed in appropriate volumes to minimise damage due to freezing and thawing.