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Development of a TaqMan PCR assay for the detection of *Bonamia* species

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ABSTRACT: The development of molecular diagnostic assays with increased sensitivity compared with conventional histological techniques is highly desirable for effective management of bonamiosis in cultured oyster stocks and wild populations. A real-time TaqMan PCR assay was developed for the specific detection of *Bonamia* species in infected oyster tissues. The TaqMan assay was shown to be significantly more sensitive than histopathology. Although a real-time TaqMan PCR assay is comparable with conventional PCR in terms of sensitivity, it offers the advantages that it is a rapid test and has a very low risk of sample cross-contamination. Furthermore, it can be optimised to quantify the parasite load in samples. The assay detected *Bonamia* isolates from Australia, New Zealand, Europe, Canada, Chile and the USA and therefore demonstrated genus specificity as tested in this study.

KEY WORDS: *Bonamia* spp. · Real-time TaqMan PCR assay · Oyster

INTRODUCTION

Diagnosis of infection with *Bonamia* species is generally achieved by means of histology, a technique currently recommended for screening purposes by the Office International des Epizooties, the World Organisation for Animal Health (OIE 2003). Histology allows topographic assessment of infection by light microscopy through direct visualisation of the parasite, as well as observation of associated lesions. However, this technique is time consuming, requires highly trained staff and has a low sensitivity (Berthe et al. 1999, da Silva & Villalba 2004). It has been suggested that low-level infections of *Bonamia* spp. may well remain undetected in oyster samples tested by such techniques (Diggles et al. 2003). While low performance of a test can usually be compensated by increasing sample size, the excessive cost associated with the processing of large numbers of samples makes this option impractical. This issue undermines the credibility of *Bonamia*-free status assessment, especially when environmental conditions are not conducive to clinical expression of the disease or when recent introduction of *Bonamia* is suspected to have occurred. Clearly, a limited ability to diagnose the presence of these parasites can impede the access of molluscs and their products to international markets. Development of improved tools for surveillance and monitoring is consequently highly desirable.

Three species of *Bonamia*, *B. ostreae* (Pichot et al. 1979, Carnegie et al. 2000, Cochennec et al. 2000), *B. exitiosa* (Dinamani et al. 1987, Hine et al. 2001, Berthe & Hine 2003) and *B. roughleyi* (Farley et al. 1988, Cochennec-Laureau et al. 2003) are currently listed by the OIE. A PCR-restriction fragment length polymorphism (PCR-RFLP) assay has been proposed to distinguish between these 3 species (Hine et al. 2001). In addition, Australian flat oysters *Ostrea angasi* are

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known to harbour *Bonamia* sp., an endemic isolate that has caused mortalities in the states of Victoria, Tasmania and Western Australia (Hine 1996, Cochennec-Laureau et al. 2003, Diggles 2003). Other isolates of *Bonamia* have been reported from *O. chilensis* in Chile (Campalans et al. 2000), *Crassostrea ariakensis* in North Carolina (Burreson et al. 2004) and *O. puelchana* in Argentina (Kroeck & Montes 2005). Taxonomic relationships between these isolates and described species within the genus need to be established.

Although recent developments of PCR-based assays have partially addressed the limitations of histology (Carnegie & Cochennec-Laureau 2004), real-time PCR (polymerase chain reaction) has the potential to provide rapid and quantitative results. In order to establish an effective diagnostic capability that will help prevent the introduction of exotic *Bonamia* spp. and to avoid the spread of enzootic isolates, the development of a molecular-based diagnostic assay that allows rapid, reliable and sensitive detection of *Bonamia* spp. is required. This report describes the development of a real-time PCR assay capable of detecting *Bonamia* isolates with greater sensitivity than currently available methods.

MATERIALS AND METHODS

Isolates of *Bonamia* spp. Wild flat oysters *Ostrea angasi*, used as a source of *Bonamia* sp.-infected tissue, were collected and fixed in 95% ethanol, during the course of a survey on the health and genetics of stocks in 5 estuaries on the south coast of New South Wales, Australia (Heasman et al. 2004). European flat oyster *O. edulis* tissues, infected with isolates of *B. ostreae* and fixed in 95% ethanol, were obtained from France (6 samples) and the Netherlands (2 samples). Four bluff oyster *O. chilensis* tissues, infected with *B. exitiosa* and fixed in 95% ethanol, were obtained from New Zealand. In addition, DNA prepared from *Bonamia*-infected oysters *O. edulis* (Canada and USA) and *O. chilensis* (Chile) was included in the study. Furthermore, DNA obtained from related haplosporidians, as well as from *Mikrocytos mackini*, was included for specificity assessment during test development.

***Bonamia* DNA extraction.** DNA from *Bonamia*-infected and uninfected oyster tissues (20 mg of gill and heart) was extracted using a QiaAmp DNA mini kit (Qiagen) according to the manufacturer's instructions. DNA bound to minicolumns was eluted and resuspended in a final volume of 100 µl of sterile deionised water. Extracted DNA was diluted 1 in 10 (corresponding to approximately 700 ng μl^{-1} of total DNA) prior to amplification in the real-time TaqMan PCR assay.

Diagnostic real-time PCR TaqMan assay. ***TaqMan primers and probe:*** An ABI Prism 7700 Sequence Detection System and Sequence Detector software Version 1.9 (PE Applied Biosystems) were 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, TaqMan MGB Probe and Primer Design (PE Applied Biosystems). The *Bonamia* primers and probe were based on the Internal Transcribed Spacer 1 (ITS-1) region of the rRNA operon, a relatively conserved genomic region among *Bonamia* isolates. Primer and probe sequences were as follows: forward primer (ITS-For): 5′-CCCTGCCCTTTGTACACACC-3′; reverse primer (ITS-Rev): 5′-TCACAAAGCTTCTAAGAACGCG-3′; and 6-carboxyfluorescein (FAM)-labelled probe (minor groove binder/non-fluorescent quencher) (Bon ITS): 6FAM-TTAGGTGGATAAGAGCCGC. The ITS primers were used at a final concentration of 900 nM each. The Applied Biosystems 18S rRNA endogenous control primers and probe were used to validate the DNA extraction procedure from oyster tissues, confirm the integrity of the extracted DNA and determine the absence of PCR inhibitors. The primers were used under limiting conditions at a final concentration of 113 nM each. The ITS FAM probe and the 18S control VIC probe were used at final concentrations of 250 and 31 nM, respectively. The reactions were carried out in 96-well plates in a 25 µl reaction volume containing 12.5 µl Universal Master Mix (PE Applied Biosystems). Aliquots (2 µl) of each DNA sample were added to the reaction mix, and the following thermal cycling conditions were used: 50°C for 2 min, 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and 63.6°C for 1 min. All reactions were repeated 3 times independently to assess the reproducibility of the results. A sample was considered positive when the change in fluorescence normalised reported (ΔR_n) of FAM or VIC, relative to that of ROX (internal reference signal), exceeded the arbitrarily set threshold values of 0.05 for FAM and 0.04 for VIC in the log phase of the amplification plots at a cycle threshold (C_T) value <35. C_T is defined as the cycle at which a statistically significant increase in fluorescence output above background is detected.

Specificity testing of the TaqMan assay: Oyster tissues used as positive and negative controls were collected from the Merimbula and Narooma estuaries in New South Wales and from Georges Bay on the east coast of Tasmania, respectively. Presence and absence of *Bonamia* infection was assessed by histology and conventional PCR assays. In order to demonstrate assay specificity, DNA preparations from phylogenetically related organisms such as *Haplosporidium nelsoni* and *H. costale* were used as negative controls. The

Table 1. Summary of semi-quantitative scheme for assessing *Bonamia* infections in *Ostrea* spp.

Host species	Light infection Score = 1	Moderate infection Score = 2	Heavy infection Score = 3
O. angasi	<10 microcells per section	10–250 microcells per section	>250 microcells per section
	1–2 microcells per infected haemocyte	>1 focus of infected haemocytes per section	Multiple foci of infected haemocytes with >2 microcells per infected haemocyte
O. edulis	1–10 microcells per section	11–50 microcells per section	>50 microcells per section

oyster parasite, *Mikrocytos mackini*, was also used as a specificity control.

Conventional PCR amplification of the rRNA partial 18S gene. The primer pair Bo/Boas, originally described by Cochennec et al. (2000), was used to amplify *Bonamia* DNA for sequencing purposes, to confirm the presence of *Bonamia* cells and to confirm the results obtained by the TaqMan assay. In order to obtain sufficient quantities of DNA for sequencing purposes, the PCR was performed a second time on each PCR product. Each PCR was conducted with a total volume of 25 µl of reaction mixture containing PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl), 1 mM $MgCl_2$, 200 µM each of dATP, dGTP, dCTP and dTTP, and 2.5 U *Taq* DNA polymerase (Promega). In all PCRs, the primers were used at a final concentration of 1 µM. Aliquots (2 µl) of extracted DNA samples were added to 23 µl of reaction mixture for the amplification. Thermocycling conditions for the PCR were as follows: 94°C for 2 min (1 cycle), followed by 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (40 cycles), followed by 72°C for 5 min (1 cycle). The resulting amplicons were resolved by electrophoresis in 2% agarose gels using 1 × TAE (0.04 M tris-acetate, 0.001 M EDTA) buffer. Gels were stained with ethidium bromide (0.5 µg ml^{-1}).

Sequence determination. The *Bonamia* 18S rRNA 300 bp partial gene sequence was determined by direct sequencing of the PCR product. Sequencing was carried out using an ABI PRISM Ready Reaction Big dye Termination Cycle Sequencing Kit (Perkin-Elmer) and an ABI PRISM Model 377XL DNA sequencer (Sequencing Analysis 3.4.1 software Version 2.6; Perkin-Elmer).

Sensitivity testing. The sensitivity of the TaqMan assay was assessed using DNA extracted from 44 *Ostrea angasi* sourced from Australia and 44 *O. edulis* from Europe, previously graded by conventional histopathology for the presence of *Bonamia* sp. and *B. ostreae* cells, respectively. Each oyster was shucked at the sampling site, a standard transverse section (5 mm thick) was excised (Howard & Smith 1983) and fixed in either 10% formalin made with filtered seawater (*O. angasi*) or in Davidson's fixative (*O. edulis*) (Bell & Lightner 1988). The samples were then transported to the pathology laboratory, where they were processed for histopathological examination using standard techniques (Howard & Smith 1983).

One section from each oyster, stained with haematoxylin and eosin (H & E), was examined for the presence of *Bonamia* spp. using a light microscope at both low and high magnifications. As no internationally recognised reference scoring method is currently available, 2 different semi-quantitative scoring methods were used by the Australian and French laboratories (Table 1).

Statistical analysis. Data were analysed using ANOVA, multiple range and Kruskal-Wallis tests (The Stat Advisor). Test results showing a value of $p < 0.05$ indicated a significant difference between means at the 95% confidence level.

RESULTS AND DISCUSSION

The *Bonamia* ITS primers and probe designed in this study identified *Bonamia* DNA specifically; when amplification was performed, a statistically significant

Table 2. Summary of results demonstrating TaqMan assay specificity. Since, for each experiment, the TaqMan assay is performed in triplicate wells, the average cycle threshold (C_T) value is given. C_T values ≥35 indicate the absence of specific amplification

Sample	Average C_T			Mean	SD
	Expt 1	Expt 2	Expt 3		
Uninfected tissue (negative control)	35.00	35.00	35.00	35.00	–
Mikrocytos mackini	35.00	35.00	35.00	35.00	–
Haplosporidium nelsoni	35.00	35.00	35.00	35.00	–
Haplosporidium costale	35.00	35.00	35.00	35.00	–
Bonamia sp. (NSW, Australia)	21.69	22.44	21.74	21.96	0.42
Bonamia exitiosa (New Zealand)	23.60	23.96	23.53	23.70	0.23
Bonamia ostreae (France)	16.20	16.26	16.91	16.46	0.39
Bonamia ostreae (Netherlands)	16.12	16.28	16.32	16.24	0.10
Bonamia ostreae (BC, Canada)	28.82	29.26	28.98	29.02	0.22
Bonamia ostreae (WA, USA)	18.82	19.54	18.69	19.02	0.45
Bonamia sp. (Chile)	20.02	19.80	19.83	19.88	0.12

Table 3. *Ostrea angasi*. Comparison of TaqMan assay with histological examination of tissues. Since, for each experiment, the TaqMan assay is performed in duplicate wells, the average C_T value is given. (Note: C_T values ≥35 indicate the absence of specific amplification.) N: negative; L: low; M: medium

Sample no.	Average C_T Expt 1	Expt 2	Expt 3	Mean	SD	Histology grading
1	27.71	28.22	29.22	28.38	0.76	N
2	26.05	26.77	27.75	26.86	0.85	N
3	30.35	30.38	32.76	31.16	1.38	N
4	25.65	25.75	26.81	26.07	0.64	N
5	22.75	22.52	23.18	22.81	0.33	N
6	28.70	28.35	29.29	28.78	0.47	N
7	27.75	28.20	28.88	28.27	0.56	N
8	34.85	34.50	34.71	34.68	0.17	N
9	34.91	34.01	34.93	34.61	0.52	N
10	32.88	31.75	32.87	32.5	0.64	N
11	34.81	34.17	35.00	34.66	0.43	N
12	33.48	33.01	34.08	33.52	0.53	N
13	32.95	32.31	33.49	32.91	0.59	N
14	32.5	31.88	33.03	32.47	0.57	N
15	30.27	30.49	31.45	30.73	0.62	N
16	28.55	28.56	29.19	28.76	0.36	N
17	35.00	35.00	35.00	35.00	–	N
18	32.42	32.6	33.48	32.83	0.56	N
19	32.22	32.97	33.17	32.78	0.50	N
20	35.00	35.00	35.00	35.00	–	N
21	30.27	31.23	31.77	31.08	0.77	N
22	25.6.0	26.6	27.64	26.61	1.02	N
23	28.66	28.82	30.10	29.19	0.78	L
24	27.75	28.26	29.38	28.46	0.83	L
25	24.7.0	25.09	26.18	25.32	0.76	L
26	25.19	25.11	25.87	25.39	0.41	L
27	23.12	22.85	23.94	23.30	0.56	L
28	21.22	20.89	21.84	21.31	0.48	L
29	27.70	27.06	27.88	27.54	0.43	L
30	28.28	27.52	28.50	28.10	0.51	L
31	27.40	26.67	27.66	27.24	0.51	L
32	32.45	32.25	33.49	32.73	0.66	L
33	27.97	27.81	28.95	28.24	0.61	L
34	21.13	20.94	21.57	21.21	0.32	L
35	28.58	29.22	30.29	29.36	0.86	L
36	31.70	32.16	33.33	32.39	0.84	L
37	30.81	30.96	32.32	31.36	0.83	L
38	24.50	25.14	25.79	25.14	0.64	L
39	26.04	25.80	27.00	26.28	0.63	M
40	21.09	20.76	22.14	21.33	0.72	M
41	28.55	29.39	30.28	29.40	0.86	M
42	29.53	30.27	30.88	30.22	0.67	M
43	24.34	25.59	26.47	25.46	1.07	M
44	22.33	22.46	23.66	22.81	0.73	M
Neg. control						
	35.00	35.00	35.00	35.00	–	–
Pos. control						
	20.95	21.14	22.36	21.48	0.76	–
Prevalence of infection				42/44 (95%)		22/44 (50%)

increase in fluorescence was recorded in the wells containing DNA from *Bonamia* isolates, regardless of the geographic origin. On the other hand, no *Bonamia*-specific product was amplified from wells containing DNA from either related organisms or uninfected oyster tissue, demonstrating specificity of the assay (Table 2).

The TaqMan assay performed on DNA extracted from *Bonamia* sp.-infected *Ostrea angasi* tissues showed that all samples assessed positive by conventional histopathology were also positive by the real-time PCR assay. In addition, out of 22 samples assessed negative by histopathology, 20 yielded positive results using the real-time PCR TaqMan assay (Table 3). The overall prevalence of infection in *O. angasi*, as determined by histopathology and real-time PCR assay, was 50 and 95%, respectively (Table 3). In order to confirm the TaqMan assay results, weak positive samples (C_T values ranging between 33 and 35) were also amplified by conventional PCR. The resulting amplicons were consequently sequenced and confirmed to be *Bonamia* specific. With regard to samples from Europe (*O. edulis* infected with *B. ostreae*), the TaqMan assay could also detect all levels of infection assessed by histopathology (Table 4).

These results are not surprising as it is well known and recognised that PCR technologies provide a higher level of sensitivity when compared with histopathology. Our data are also consistent with results previously obtained, which show that PCR was more sensitive than the use of heart imprints and histology for the detection of *Bonamia ostreae* and *B. exitiosa* (Carnegie et al. 2000, Cochennec et al. 2000, Diggles et al. 2003).

Although the TaqMan assay detected the presence of *Bonamia* in *Ostrea angasi* that had been judged 'negative' for the presence of *Bonamia* by histopathology, no epizootics or large, unexplained mortality events of farmed oysters have been reported from any estuary of southern New South Wales in the last 5 yr. This observation contrasts with reports of flat oyster mortalities that have occurred in other Australian states (Hine 1996). Further investigation would be required to shed light on virulence differences between Australian *Bonamia* isolates and host-species specificity, as well as epizootiological factors such as the effects of water temperature, oyster density and environmental stressors on *Bonamia* infections and bonamiosis disease.

With regard to the level of infection in each oyster sample, it is noteworthy that there was an inverse correlation between histology values (negative, low and medium) and C_T values from the TaqMan assays. Samples infected with higher numbers of *Bonamia* cells were detected more rapidly (low C_T values) than sam-

ples containing fewer *Bonamia* cells (high C_T values) (Table 5). Similarly, *Ostrea edulis* samples containing higher loads of *B. ostreae* showed a smaller average C_T value than those containing a lower number of parasite cells (Table 6). This is to be expected as a higher load of *Bonamia* cells provides a larger amount of DNA target, hence, allowing the parasites to be detected sooner in the PCR amplification process.

Table 4. *Ostrea edulis*. Comparison of TaqMan assay with histological examination of tissues. Since, for each experiment, the TaqMan assay is performed in duplicate wells, the average C_T value is given. (Note: C_T values ≥35 indicate the absence of specific amplification.) N: negative; L: low; M: medium

Sample no.	Average C_T Expt 1	Expt 2	Expt 3	Mean	SD	Histology grading
1	34.16	34.84	34.37	34.46	0.34	L
2	33.80	34.22	34.76	34.26	0.48	L
3	32.27	32.12	32.62	32.34	0.26	L
4	32.16	32.63	32.40	32.40	0.23	L
5	27.66	27.75	27.88	27.76	0.11	L
6	27.72	27.83	28.40	27.98	0.36	L
7	27.07	27.10	27.64	27.27	0.32	L
8	27.52	27.48	27.66	27.55	0.09	L
9	30.24	30.28	29.91	30.14	0.20	L
10	30.20	30.43	30.16	30.26	0.15	L
11	26.19	25.96	25.51	25.89	0.35	L
12	26.19	26.30	25.91	26.13	0.20	L
13	28.64	27.68	28.51	28.28	0.52	L
14	28.45	28.26	27.88	28.20	0.29	L
15	21.99	22.10	22.08	22.06	0.06	L
16	21.99	22.05	22.04	22.03	0.03	L
17	22.88	21.78	22.50	22.39	0.56	M
18	22.76	22.26	22.43	22.48	0.25	M
19	27.30	27.30	27.45	27.35	0.09	M
20	27.65	27.42	27.57	27.55	0.12	M
21	26.24	28.49	25.92	26.88	1.40	M
22	26.86	26.18	26.00	26.35	0.45	M
23	27.68	27.86	27.53	27.69	0.16	M
24	27.35	27.49	27.60	27.48	0.12	M
25	24.17	24.34	24.17	24.23	0.10	M
26	23.98	24.16	24.07	24.07	0.09	M
27	20.36	20.45	20.40	20.40	0.05	M
28	20.31	20.53	20.42	20.42	0.11	M
29	20.42	20.18	20.48	20.36	0.16	H
30	20.46	20.49	20.54	20.50	0.04	H
31	26.56	26.10	25.88	26.18	0.34	H
32	26.92	26.33	26.33	26.53	0.34	H
33	21.68	21.32	21.14	21.38	0.28	H
34	21.36	21.51	21.32	21.40	0.10	H
35	19.82	19.18	19.34	19.45	0.33	H
36	19.22	19.16	19.09	19.16	0.07	H
37	21.08	20.06	20.26	20.47	0.54	H
38	20.65	20.27	20.3	20.41	0.21	H
39	22.53	22.4	23.15	22.69	0.40	H
40	22.44	22.49	22.59	22.51	0.08	H
41	20.43	20.26	20.29	20.33	0.09	H
42	20.6	20.47	20.61	20.56	0.08	H
43	21.22	21.26	21.57	21.35	0.19	H
44	21.28	21.43	21.74	21.48	0.23	H
Neg. control						
	35.00	35.00	35.00	35.00	–	–
Pos. control						
	22.1	22.02	25.72	23.28	2.11	–

The TaqMan assay presented in this study has the added advantages, compared to more traditional histopathological assays, that it is highly sensitive, specific, rapid and allows high throughput. The cost of running the assay is approximately half the cost of histopathology diagnosis; it provides a more correct assessment of the prevalence of the pathogen and reduces the potential for false 'negatives'. Indeed, many tissue samples assessed as negative by histopathology yielded positive results using the TaqMan assay. In addition, the TaqMan assay presents a very low risk of sample cross-contamination and can be optimised to quantify the parasite load in samples. Also, large numbers of oysters can be nondestructively screened (Carnegie et al. 2000).

However, PCR-based assays have limitations. Inhibitory factors in mollusc tissues may contribute to false negative results. In addition, degraded pathogen DNA may generate positive results where there are no viable parasites. Furthermore, it is noted that light infections may be missed due to the small size of the tissue samples; however, this could have a limited impact, as the infection with *Bonamia* species is usually systemic, with infectious particles being transported by haemocytes.

Table 5. *Ostrea angasi*. Comparison of mean C_T values per group of oysters with histological evaluation. Mean C_T values were obtained from the mean values of each histology-graded group presented in Table 3

Mean C_T	SD	Histology grading
30.93[a]	3.41	Negative
27.27[b]	3.50	Low
25.92[b]	3.51	Medium

[a]Significantly different from other means ($p < 0.01$)
[b]Low and medium mean C_T values are not significantly different from each other

Table 6. *Ostrea edulis*. Comparison of mean C_T values per group of oysters with histological evaluation. Mean C_T values were obtained from the mean values of each histology-graded group presented in Table 4

Mean C_T	SD	Histology grading
28.56[a]	3.68	Low
24.77[a]	2.81	Medium
21.55[a]	2.09	Heavy

[a]Significantly different from other means ($p < 0.01$)

The results obtained in our study demonstrate that the TaqMan assay provides a useful complement to the conventional methods currently used for the detection of *Bonamia*. It is anticipated that, with wider use of this TaqMan real-time PCR assay, improved surveillance will be achieved, thereby benefitting epidemiological studies, which should lead to improved management and prevention of epizootics in both wild and farmed oysters.

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