

Species-specific oligonucleotide probe for detection of *Bonamia exitiosa* (Haplosporidia) using *in situ* hybridisation assay

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ABSTRACT: Bonamiosis is a disease affecting various oyster species and causing oyster mass mortalities worldwide. The protozoans *Bonamia exitiosa* and *B. ostreae* (Haplosporidia) are included in the list of notifiable diseases of the World Organisation for Animal Health as the causative agents of this disease. Although the geographic range of both species was considered different for years, both species are now known to co-occur in some European areas affecting the same host, *Ostrea edulis*, which strengthens the need of species-specific methods to unequivocally identify the species of *Bonamia*. An oligonucleotide probe for specific detection of *B. exitiosa* (BEX_ITS) was designed to be used in *in situ* hybridisation (ISH) assays. ISH assay with BEX_ITS probe showed species-specificity and more sensitivity than traditional histology to visualise the parasite inside host tissue. ISH assay showed that the oyster gonad was the area where the parasite was most frequently located, and was the exclusive organ of infection in some oysters. A recommendation arising from the study is that more than 1 organ (including gonad and gills) should be used for PCR-based diagnosis of *B. exitiosa*, to maximise the sensitivity.

KEY WORDS: Bonamiosis · *Ostrea edulis* · Diagnostic assay · Specificity · Diagnosis

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INTRODUCTION

Bonamia exitiosa is a protozoan parasite belonging to the phylum Haplosporidia, which infects various species of oysters, causing extensive mortalities worldwide. *B. exitiosa* or *Bonamia* sp. resembling *B. exitiosa* have been described infecting *Ostrea chilensis* from New Zealand and Chile (Dinamani et al. 1987, Campalans et al. 2000, Berthe & Hine 2003), *O. angasi* from Australia (Hine & Jones 1994, Corbeil et al. 2006a), *O. puelchana* from Argentina (Kroeck & Montes 2005) and *Crassostrea ariakensis* from the USA (Burreson et al. 2004). Bonamiosis in Europe had been solely attributed to the species *B. ostreae* parasitising the flat oyster *O. edulis* (Pichot et al. 1980, Bucke et al. 1984, Montes & Melendez 1987, McArdele et al. 1991, da Silva et al. 2005), until Abollo

et al. (2008) reported the co-occurrence of 2 *Bonamia* species, namely *B. ostreae* and *B. exitiosa*, infecting *O. edulis* in NW Spain. Subsequently, *B. exitiosa* was also reported parasitising *O. edulis* in Italy (Narcisi et al. 2010) and France (Arzul et al. 2011) and infecting *O. stentina* in Tunisia (Hill et al. 2010). *B. ostreae* and *B. exitiosa* are included in the list of notifiable diseases of the World Organisation for Animal Health (OIE) (www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2012/) and the lists of diseases requiring surveillance by the European Regulation on Animal Health (Council of the European Union 2006).

Traditionally, detection of *Bonamia* in oyster tissue involved microscopic examination of histological sections and examination of gill or heart tissue imprints. However, these methods show low sensitivity and

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specificity (O'Neill et al. 1998, Culloty et al. 2003, Diggle et al. 2003, da Silva & Villalba 2004, Balseiro et al. 2006, Marty et al. 2006, Lynch et al. 2008), are time-consuming and require a trained observer to screen the samples, because the small size makes parasite detection difficult in cases of very light infections, and the morphological similarity between *Bonamia* species hinders species identification (Katkansky et al. 1969, Balouet et al. 1983, Bucke et al. 1984, Elston et al. 1986, Friedman et al. 1989, Hine et al. 2001, Abollo et al. 2008, Lohrmann et al. 2009). Using molecular methods, such as those based on the polymerase chain reaction (PCR) and *in situ* hybridisation (ISH) with DNA probes, increases the sensitivity. Nevertheless, the reported PCR assays (Carnegie et al. 2000, 2006, Cochenne et al. 2000, Abollo et al. 2008) are not species-specific. Due to this limitation, specific identification of the microcells has been achieved by restriction fragment length polymorphism (RFLP) assays (Hine et al. 2001, Cochenne-Laureau et al. 2003, Abollo et al. 2008) or sequencing the PCR products. Real-time PCR assays for the identification of *Bonamia* spp. were also developed by Marty et al. (2006) and Corbeil et al. (2006b). Recently, PCR assays for specific detection of *B. ostreae* and *B. exitiosa* have been developed. Robert et al. (2009) and Engelsma et al. (2010) designed specific primers to identify *B. ostreae* by conventional PCR assay, whereas Ramilo et al. (2013) developed a real-time PCR and a conventional PCR assay for the specific identification of *B. exitiosa* and *B. ostreae*. Moreover, they designed a multiplex PCR assay able to detect both parasites simultaneously in a unique assay.

As highlighted by Burreson (2008), when using PCR assays for parasite detection, positive results do not necessarily always correspond with actual infections. ISH assays for the detection of *Bonamia* species on histological sections have also been developed. Cochenne et al. (2000) designed a polynucleotide probe, named Bo/Boas based on the small subunit ribosomal DNA (SSU rDNA) gene sequence of *B. ostreae*. Nevertheless, the probe is not species-specific, showing cross-reaction with *B. exitiosa* and *Haplosporidium nelsoni*. A fluorescent ISH assay developed by Carnegie et al. (2003), using a cocktail of 3 probes, is also not species-specific, because hybridisation with *B. ostreae* and *B. exitiosa* can occur. Recently, Hill et al. (2010) developed another cocktail of 3 digoxigenin (DIG)-labelled probes specific for closely related members of the *B. exitiosa*–*B. roughleyi* clade; however, neither specificity nor sensitivity of the oligonucleotide probes was evaluated in that paper.

Having a tool with high sensitivity and species-specificity to visualise the parasites in the host tissues would be useful for epidemiological studies in areas where 2 or more *Bonamia* species co-occur (Abollo et al. 2008, Narcisi et al. 2010, Arzul et al. 2011). Thus, the aim of this study was to establish a species-specific detection method for *B. exitiosa* by ISH. The design of a DNA probe and the validation of its use in ISH assays are reported.

MATERIALS AND METHODS

Oyster sampling

A total of 30 flat oysters *Ostrea edulis* were used in this study. Ten oysters had been collected in May 2007 and 20 oysters in June 2007 from a commercial raft located in Cambados, Ría de Arousa (Galicia, NW Spain), an area affected by *Bonamia ostreae* and *B. exitiosa* (Polanco et al. 1984, da Silva et al. 2005, Abollo et al. 2008).

Design and synthesis of probes

A first attempt was made using SSU rDNA as the target gene for probe design. Due to the high similarity of this gene (96–97%) among *Bonamia* species, the ITS1 region was selected to design the oligonucleotide probe. Sequences of this region from other haplosporidian species were not available in GenBank. The possible segments of interest were selected by alignment of 119 sequences belonging to the genus *Bonamia*, using the program ClustalW. The alignment comprised *B. ostreae* (EU709105–11, AF262995, AF162087), *B. perspora* (EU709112–32), *Bonamia* sp. from *Crassostrea ariakensis* (USA) (EU709024–54), *Bonamia* sp. from *Ostrea angasi* (Australia) (EU723225–31), *B. exitiosa* (New Zealand) (EU709069–75), *Bonamia* sp. from *O. puelchana* (Argentina) (EU709055–68), and *Bonamia* sp. from *O. chilensis* (Chile) (EU709076–104). The oligonucleotide probe was designed using the program Primer 3 (Rozen & Skaletsky 2000). Subsequently, the probe sequence was submitted to the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/blast.cgi) to ensure probe specificity to *B. exitiosa* and to exclude unintended cross-hybridisation with other organisms. The probe (BEX ITS) was commercially synthesized (TIB MOLBIOL) and was 3' end-labelled with DIG.

A Bo/Boas polynucleotide probe (Cochenne et al. 2000) was also synthesized in order to be used as a

positive control in some ISH assays. A PCR DIG ProbeSynthesis kit (Roche Applied Science) was used to amplify a fragment of DNA of *Bonamia* spp., with the primer pair Bo/Boas described by Cochenec et al. (2000), labelled with DIG by incorporation of DIG-dUTP during the PCR process.

Histology and ISH assay

A frontal section (5 mm) of the meat of each oyster containing gills, visceral mass and mantle lobes was fixed in Davidson's solution, dehydrated in an ethanol series and embedded in paraffin. Four serial tissue sections (5 µm) were made: 1 section was used for testing the BEX_ITS probe, another section was used as a positive control for ISH, employing the generic probe Bo/Boas for *Bonamia* spp., the third section was used as the negative control (without probe), and the last section was stained with Harris' haematoxylin and eosin (H&E) in order to identify and localise the parasite. Specific identification of *B. exitiosa* cells was performed according the morphological distinguishing criteria reported by Abollo et al. (2008). Each oyster was ranked according to the following scale of infection intensity: no infection (0), when no parasite was detected; light infection (1), when only 1 focus of haemocyte infiltration was observed and no more than 10 parasites were detected in the focus; moderate infection (2), when various foci of haemocytic infiltration with parasites were observed in the section, sometimes in various organs, or when only 1 large focus was observed with more than 10 parasites; and heavy infection (3), when numerous parasites occurred throughout the connective tissue of multiple various organs (gonads, digestive gland, gills, mantle, labial palps), frequently in extracellular locations.

Sections for ISH assays were placed on silanized slides (Silane-prep slides; Sigma) and incubated overnight at 55°C. The sections were deparaffinised in xylene for 5 min, repeating once and rehydrating in an ethanol series. Sections were permeabilised with Proteinase K (50 µg ml⁻¹) in PBS buffer at 37°C for 10 min in a humid chamber. Proteolysis was inactivated by washing in PBS with 0.2% glycine, and sections were incubated with 500 µl of hybridisation buffer (MicroHyb, Invitrogen) at 42°C for 30 min and air dried. Samples were covered with *in situ* plastic coverslips (Sigma) and treated with 100 µl of hybridisation buffer containing (1) 200 ng of the probe BEX_ITS, (2) 50 ng of the probe Bo/Boas (positive control), or (3) no probe (negative control). Slides were kept at 80°C for 10 min to denature the target

DNA and then cooled on ice for 5 min, before hybridisation at 42°C, overnight in a humid chamber. After this, *in situ* plastic coverslips were removed and sections were washed twice for 5 min in 2× saline sodium citrate (SSC) at room temperature and once for 10 min in 0.4× SSC at 42°C. Subsequently, a DIG Nucleic Acid Detection Kit (Roche Applied Science) was used for the immunological detection of the DIG-labelled probes. The slides were washed for 2 min in DIG 1 buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20, pH 7.5) and incubated for 30 min at room temperature (RT) with 200 µl of blocking solution DIG 2 (10% blocking reagent in maleic acid buffer) in a humid chamber. After this, sections were incubated for 1 h at RT with 200 µl of the antibody antidigoxigenin-AP diluted in DIG 2 buffer 1:1000. Slides were washed twice for 15 min in DIG 1 and incubated for 5 min in detection buffer DIG 3 (0.1 M Tris HCl, 0.1 M NaCl, pH 9.5). Next, the slides were incubated for 2 h with 200 µl of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl (NBT/BCIP) solution diluted (2%) in DIG 3 buffer. Incubations were carried out in a humid chamber in the dark. Reactions were stopped in distilled water for 2 min, and then slides were stained for 7 min in Bismark brown Y, dehydrated with ethanol and mounted with Histomount (Zymed, Invitrogen).

Analytical specificity of the BEX_ITS probe

The analytical specificity of the BEX_ITS probe was assessed using tissue sections of flat oysters *Ostrea edulis* parasitised by *Bonamia exitiosa*, *B. ostreae*, *Haplosporidium armoricanum* and *Marteilia refringens*; a Pacific oyster *Crassostrea gigas* infected with *H. nelsoni*; a carpet-shell clam *Ruditapes decussatus* infected with *Perkinsus olseni*; and a Sydney rock oyster *Saccostrea glomerata* parasitised by *M. sydneyi*. Three serial tissue sections (5 µm) were made: the first section was used for testing the BEX_ITS probe, the second section was used as a negative control (without probe), and the last section was stained with H&E.

Comparison of ISH with other diagnostic techniques

In order to assess the sensitivity of the new ISH assay, 30 oysters were diagnosed with the new species-specific ISH for *Bonamia exitiosa* and the results were compared to diagnoses with 2 OIE-recommended techniques: standard histology and the PCR-RFLP assay described by Cochenec-Laureau et al.

(2003). Additionally, species-specific conventional PCR (cPCR) and real-time PCR for *B. exitiosa* described by Ramilo et al. (2013) were included in the comparison.

DNA extraction

To carry out all PCR assays, small pieces of gills of each oyster were preserved in 96% ethanol and, subsequently, DNA extractions were performed employing the commercial kit Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol to isolate genomic DNA from animal tissue. DNA quality and quantity were checked in a spectrophotometer Nanodrop® ND-1000 (Nanodrop Technologies).

PCR-RFLP

PCR was performed in a total volume of 25 µl containing 1 µl of genomic DNA (200 ng), PCR buffer at 1× concentration, 1.5 mM MgCl₂, 0.2 mM nucleotides (Roche Applied Science), 0.3 µM primers Bo/Boas (Cochennec et al. 2000) and 0.025 U µl⁻¹ *Taq* DNA polymerase (Roche Applied Science). The PCR was carried out in a TGradient thermocycler (Biometra) using the following cycling protocol: 94°C for 2 min; 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; followed by 72°C for 7 min. The RFLP assay was performed with positive PCR products. All restriction reactions were carried out in a final volume of 15 µl containing 2400 ng of DNA, 1.5 µl of enzyme buffer and 0.5 µl (5 U) of *Bgl*I restriction enzyme (Takara Bio). The digestions were performed for 3 h at 37°C, which was followed by 20 min at 65°C to inactivate the enzyme. To visualise the restriction patterns, digested samples (15 µl) mixed with 1 µl loading buffer were subjected to electrophoresis through 2% agarose gels in 1% Tris acetate EDTA buffer, stained with ethidium bromide and run at 60 V for 2 h. A 50 bp ladder (Roche Applied Science) was included as a molecular weight marker. The gels were scanned in a GelDoc XR documentation system (BioRad).

Specific cPCR for *Bonamia exitiosa*

PCR reactions were performed as described above, in this case using the primer pair BEXIT-R/BEXIT-F (Ramilo et al. 2013). The cycling protocol was 94°C

for 2 min; 35 cycles of 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 1 min. After PCR, 10 µl of amplified DNA were analysed by electrophoresis on 2% agarose as described above.

Specific real-time PCR for *Bonamia exitiosa*

Real-time PCR assays were carried out on an iQTM5 Multicolour Real-Time PCR Detection System (Bio-Rad Laboratories) using the DNA-binding dye technique (SYBR Green). Reactions were performed in a total volume of 25 µl comprising 1 µl of genomic DNA (200 ng), 12.5 µl of FastStart Universal SYBR Green Master (Rox) from Roche Applied Science, 0.3 µM primers BEXIT-F and BEXIT-R and 10 µl of distilled water. Negative control (no DNA) and positive controls (samples positive for *B. exitiosa*) were included in each PCR assay. The amplification program used for *B. exitiosa* was 95°C for 10 min; and 35 cycles of 95°C for 30 s, 58°C for 45 s and 72°C for 1 min. In order to establish the specificity of the amplicons, melting curves were established in temperature increments of 0.5°C s⁻¹ starting at 58°C and ending at 95°C. The resulting data were analysed using the software provided by the iQ5 system (Bio-Rad iQTM5 2.0 Standard Edition Optical System Software).

Statistical analysis

Sensitivity and specificity of each technique used for diagnosis of *Bonamia exitiosa* were estimated by the maximum likelihood method using the TAGS program (Pouillot et al. 2002), assuming that none could be considered as a 'gold standard.' That approach was implemented through the website www.epi.ucdavis.edu/diagnostictests/QUERY.HTM, created by the Graduate Group in Epidemiology (School of Veterinary Medicine, University of California Davis, USA). With this approach, the 5 diagnostic tests used (ISH, histology, PCR-RFLP, cPCR and real-time PCR) were compared considering that the 30 oysters corresponded to 2 different groups, viz. the sample from May and that from June 2007.

Additionally, the agreement between pairs of diagnostic tests was estimated by calculating Cohen's kappa coefficient (Cohen 1960) through the website www.winepi.net, created by Ignacio de Blas (Faculty of Veterinary, University of Zaragoza, Spain). The agreement was ranked using the following scale of the kappa coefficient (κ) (Landis & Koch 1977):

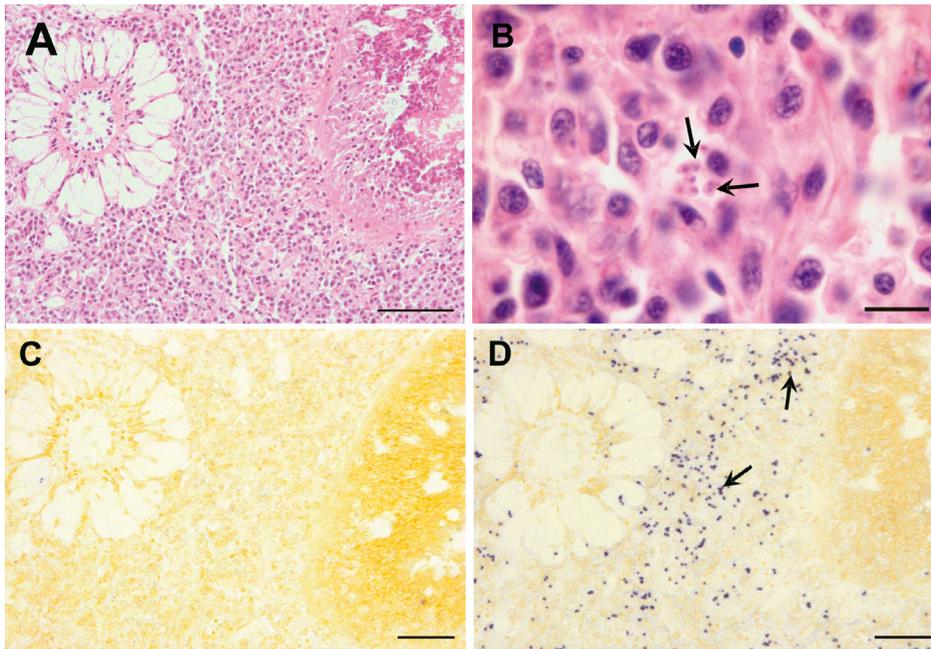


Fig. 3. *Ostrea edulis* infected with *Bonamia ostreae*. Serial histological sections. (A) H&E stained section in which *B. ostreae* is not discernible (scale bar = 50 µm). (B) H&E stained section showing *B. ostreae* (arrows) inside haemocytes of *O. edulis* (scale bar = 10 µm). (C) *In situ* hybridisation (ISH) assay with BEX_ITS probe; no hybridisation signal is observed, evidencing that *B. ostreae* is not recognised by this probe (scale bar = 50 µm). (D) Positive control for ISH using the Bo/Boas generic probe; the blue deposits (arrows) are hybridisation signals corresponding to *B. ostreae* (scale bar = 50 µm)

when the assay was made without probe (negative control, Fig. 2D).

The analytical specificity of the BEX_ITS probe was demonstrated with tissue sections of flat oyster infected by *Bonamia ostreae*, since the oligonucleotide probe did not hybridise with this *Bonamia* species (Fig. 3C), whereas the probe Bo/Boas (positive control) clearly labelled the parasite (Fig. 3D). Cross-reactivity was not observed in the ISH assays with other para-

sites: *Haplosporidium armoricanum* (Fig. 4B), *H. nelsoni* (Fig. 4D), *Marteilia refringens* (Fig. 5B), *M. sydneyi* (Fig. 5D) and *Perkinsus olseni* (Fig. 5F).

Comparison of diagnostic tests

Of 30 oysters, 25 were identified as infected with *Bonamia exitiosa* by at least 1 diagnostic test, but

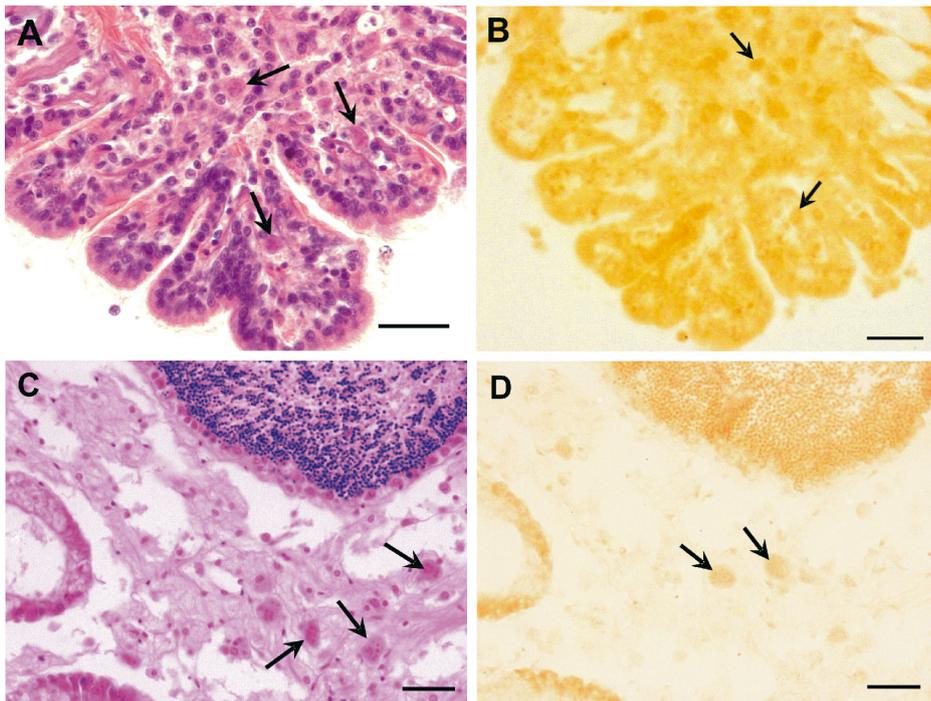


Fig. 4. *Ostrea edulis* and *Crassostrea gigas* infected with *Haplosporidium* spp. Pairs of serial histological sections of 2 molluscs used to test the specificity of the BEX_ITS probe (scale bars = 25 µm). (A) H&E stained section of *O. edulis* infected with *H. armoricanum* plasmodia (arrows) and (B) *in situ* hybridisation (ISH) assay with the BEX_ITS probe on a serial section, showing no hybridisation signal in *H. armoricanum* plasmodia (arrows). (C) H&E stained section of *C. gigas* infected with *H. nelsoni* plasmodia (arrows) and (D) ISH assay with the BEX_ITS probe on a serial section, showing no hybridisation signal in *H. nelsoni* plasmodia (arrows)

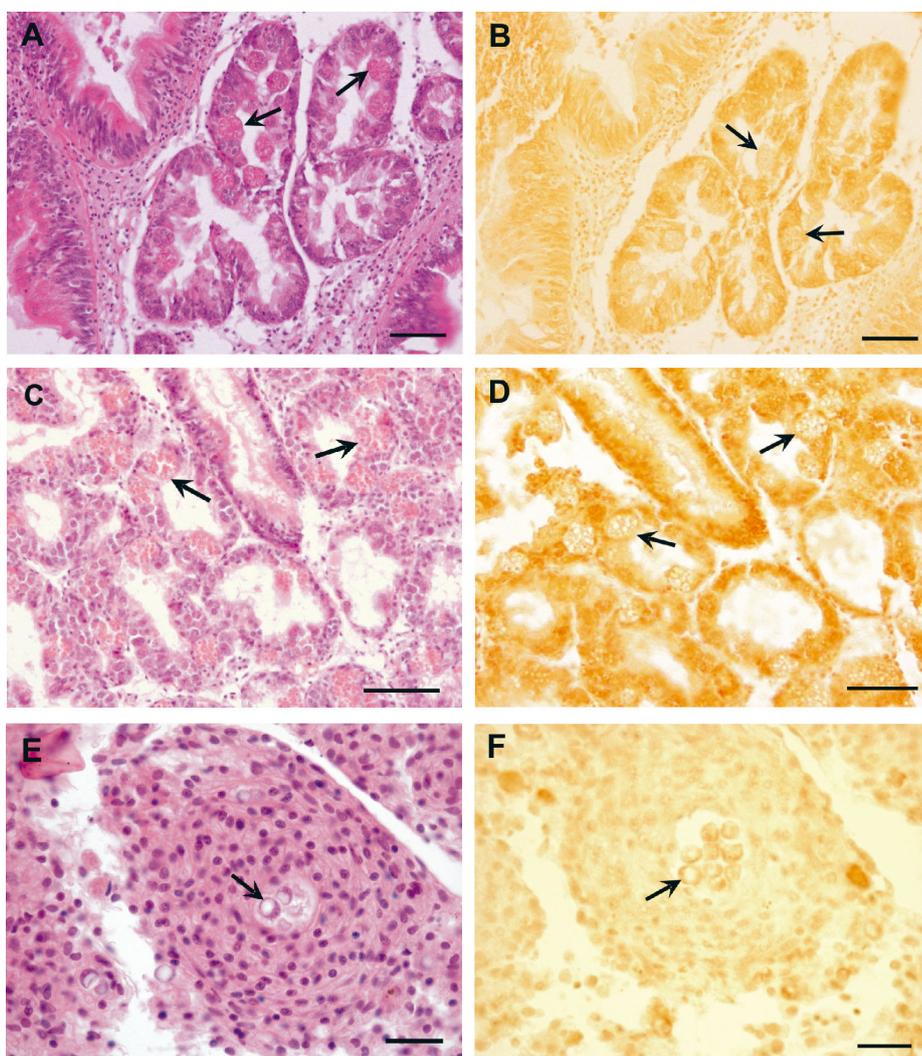


Fig. 5. Pairs of serial histological sections of 3 molluscs used to test the specificity of the BEX_ITS probe. (A) H&E stained section of *Ostrea edulis* infected with *Marteilia refringens* (arrows; scale bar = 50 µm) and (B) *in situ* hybridisation (ISH) assay with the BEX_ITS probe on a serial section, showing no hybridisation signal in *M. refringens* (arrows; scale bar = 50 µm). (C) H&E stained section of *Saccostrea glomerata* infected with *Marteilia sydneyi* (arrows; scale bar = 50 µm) and (D) ISH assay with the BEX_ITS probe on a serial section, showing no hybridisation signal in *M. sydneyi* (arrows; scale bar = 50 µm). (E) H&E stained section of *Ruditapes decussatus* infected with *Perkinsus olseni* (arrow; scale bar = 25 µm) and (F) ISH assay with the BEX_ITS probe on a serial section, showing no hybridisation signal in *P. olseni* (arrow; scale bar = 25 µm)

only 12 oysters were identified as being infected by every test (Table 1). ISH assay detected the same number of positive cases of *B. exitiosa* as the PCR-RFLP assay (19/30) recommended by the OIE (Cochennec-Laureau et al. 2003), although those diagnostic tests disagreed for 4 oysters. Both diagnostic tests detected fewer cases than species-specific cPCR and real-time PCR (22/30 and 24/30, respectively). Histology was the test with the fewest positive cases (15/30). Every oyster found infected by ISH was also found positive by at least 1 of the other tests. Remarkably, 1 oyster (no. 8 in Table 1) was found positive by ISH and histology (light intensity) but negative by the 3 PCR-based methods. Five of the 11 cases found negative by ISH were also negative in the other tests, while 1 oyster (no. 14) found negative by ISH was positive in the other 4 tests.

Positive ISH signals were observed in the connective tissue of gonads (16/19), gills (9/19), mantle lobes

(5/19) and digestive gland (5/19) (Table 2). Moreover, the intensity of infection was also higher in the gonads than in the other infected organs. In 6 oysters, hybridisation was exclusively observed in the gonads, while exclusive hybridisation in the gills occurred in 1 oyster, and no case of exclusive hybridisation in either mantle lobes or digestive gland was found. Considering the 7 oysters (nos. 2, 11, 13, 19, 22, 25 and 26) in which *Bonamia exitiosa* was detected with ISH but not with histology, which therefore were assumed to be very lightly infected, hybridisation signals were observed in the gonad of 6 of them, in the gills of 4, in the digestive gland of 3 and in the mantle of 2; hybridisation was exclusively observed in the gonads of 3 of these 7 oysters. Oyster no. 8, which had a positive diagnosis through histological sections (ISH and histology) but negative with the 3 PCR-based tests, showed a hybridisation signal exclusively in the gonads.

Table 1. *Ostrea edulis*. Results of diagnostic assays testing for the presence of *Bonamia exitiosa* in 30 oysters collected in Spain. ISH: *in situ* hybridisation

Oyster ID	ISH	Histo-logy	PCR-RFLP	Specific PCR cPCR	Specific PCR qPCR
May 2007 sample					
1	-	-	-	-	-
2	+	-	-	+	+
3	-	-	-	-	-
4	-	-	-	-	-
5	+	+	+	+	+
6	-	+	-	+	+
7	+	+	+	+	+
8	+	+	-	-	-
9	-	-	-	-	-
10	-	-	-	-	-
June 2007 sample					
11	+	-	+	+	+
12	+	+	+	+	+
13	+	-	+	+	+
14	-	+	+	+	+
15	+	+	+	+	+
16	-	-	-	-	+
17	-	-	-	-	+
18	+	+	+	+	+
19	+	-	+	+	+
20	+	+	+	+	+
21	+	+	+	+	+
22	+	-	+	+	+
23	-	-	+	+	+
24	+	+	+	+	+
25	+	+	+	+	+
26	+	-	+	+	+
27	+	+	+	+	+
28	-	-	-	+	+
29	+	+	+	+	+
30	+	+	+	+	+
Total positive	19	14	19	22	24

The values of sensitivity and specificity of the diagnostic tests estimated by the maximum likelihood method are shown in Table 3. The sensitivity values of ISH and histology were below 0.90, whereas those of PCR-RFLP, species-specific cPCR and real-time PCR were higher, reaching 1.00 in the cases of cPCR and real-time PCR. The specificity value of ISH was equal to that of histology (0.88). The highest specificity values corresponded to PCR-RFLP and species-specific cPCR (1.00), while real-time PCR showed a value lower than ISH (0.75). ISH showed moderate agreement with histology and species-specific real-time PCR and substantial agreement with PCR-RFLP and species-specific cPCR for diagnosis of *Bonamia exitiosa* (Table 4). Histology showed fair to moderate agreement with the other tests. The only case of almost perfect agreement was reached between species-specific cPCR and real-time PCR.

Table 2. Organ distribution of the hybridisation signals detected with the *in situ* hybridisation assay, considering only the positive cases

Oyster ID	Gonads	Gills	Mantle lobes	Digestive gland
2	+	-	-	-
5	+	-	-	-
7	+	+	-	-
8	+	-	-	-
11	+	-	-	-
12	+	+	+	+
13	+	+	+	+
15	+	-	-	-
18	+	+	-	-
19	-	+	+	-
20	+	-	-	-
21	-	-	+	+
22	+	+	-	+
24	+	-	-	-
25	+	-	-	-
26	+	+	-	+
27	+	+	+	-
29	+	-	-	-
30	-	+	-	-
Total positive	16	9	5	5

Table 3. Values of sensitivity (Se) and specificity (Sp) of each diagnostic test for *Bonamia exitiosa*, estimated by the maximum likelihood method using the TAGS program. ISH: *in situ* hybridisation

	ISH	Histology	PCR-RFLP	Specific PCR cPCR	Specific PCR qPCR
Se	0.82	0.63	0.91	1.00	1.00
Sp	0.88	0.88	1.00	1.00	0.75

Table 4. Values of Cohen's kappa coefficient of agreement between pairs of diagnostic tests for *Bonamia exitiosa*. ISH: *in situ* hybridisation

	Histology	PCR-RFLP	Specific PCR cPCR	Specific PCR qPCR
ISH	0.47	0.71	0.62	0.44
Histology		0.47	0.40	0.27
PCR-RFLP			0.77	0.60
cPCR				0.82

DISCUSSION

We designed a new DIG-labelled oligonucleotide probe for the detection of *Bonamia exitiosa* in tissue sections of flat oyster by ISH, thereby providing a species-specific molecular tool to unequivocally

identify this parasite and visualise it in host tissues even in geographic areas where *B. ostreae* also affects the same host. Burreson (2008) emphasised the importance of confirming by ISH assays the positive results obtained by PCR, in order to avoid false positive results, as well as the negative results obtained by histology, since ISH is more sensitive to detect slight infections.

When the analytical sensitivity of the probe BEX ITS was checked through ISH, it allowed the specific detection of *Bonamia exitiosa* but it did not recognise any other tested parasite, including *B. ostreae* and other haplosporidians, and did not cross-react with the tissues of any host. The higher level of specificity of the BEX ITS probe compared with the polynucleotide probe Bo/Boas might be due to the fact that the ITS region of the rDNA gene cluster is more suitable to develop specific molecular targets, since it presents a higher interspecific variability than the more conserved SSU rDNA region on which the probe Bo/Boas is based (Hillis & Dixon 1991, Blair & Barker 1993, Gasser 1999, Kleeman et al. 2002).

Sensitivity and specificity of the ISH assay could not be accurately estimated in the absence of a gold standard diagnostic test. Nevertheless, with the statistical approach used in the study, the estimated sensitivity of the ISH assay for *Bonamia exitiosa* was higher than that of the standard histology test recommended by the OIE. The parasite can go unnoticed in light infections due to its small size using standard histology, but the ISH assay facilitates the observation of the parasite. Thus, the agreement between both techniques was moderate. The maximum likelihood statistical approach conferred the same specificity for ISH and histology, which was <1 due to 1 case of positive detection with these 2 tests that was negative with the 3 PCR-based tests; the statistical treatment likely assumed this case to be a false positive for ISH and histology tests, but that assumption was wrong. A likely explanation is that the infection was confined to the gonads and thus could not be detected in the piece of gills processed for the PCR-based tests. The estimated sensitivity of the ISH assay was similar to that of PCR-RFLP, another OIE-recommended test for this parasite, but the estimated specificity of the latter was higher, likely due to the above mentioned reason. Both techniques showed substantial agreement. The species-specific cPCR and real-time PCR developed by Ramilo et al. (2013) showed the highest sensitivity.

Hybridisation signals were more frequent in gonads than in other organs. Furthermore, hybridisation occurred exclusively in gonads in a remarkable

number of oysters. In the case of the oysters thought to be very lightly infected (infection detected with ISH but not with histology), hybridisation was again more frequent in the gonads, but some signals were detected in the other organs in some oysters. Therefore, a preference for the gonad area could be deduced for *Bonamia exitiosa*. Such a gonad preference has not been detected for *B. ostreae* (A. Villalba & M. J. Carballal pers. obs.), although its occurrence in the gonad of its type host *Ostrea edulis* was reported by van Banning (1990), and PCR-positive results for *B. ostreae* were found in gonads of brittle stars *Ophiothrix fragilis* (Lynch et al. 2007). Likely, the number of cases of infection detected by the 3 PCR based methods tested in the study could have been higher if gonad pieces had been included for PCR diagnosis. The presence of *B. exitiosa* in the gonads has been described in other host species, namely *O. chilensis* (Hine 1991) and *O. puelchana* (Kroeck et al. 2008). Hine (1991) observed that the presence of *B. exitiosa* in the gonads was more frequent than in other organs (gills, gut, digestive gland and kidney) in the 4 seasons of the year. An obvious recommendation is that more than 1 organ (including gonads and gills) should be used for PCR-based diagnosis of *B. exitiosa*, to maximise the sensitivity of the assay.

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