Molecular detection and quantification of the protozoan *Bonamia ostreae* in the flat oyster, *Ostrea edulis*

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Abstract:

Bonamia ostreae is an intracellular protozoan which is recognized as a cause of mortality in European populations of flat oysters (*Ostrea edulis*). Based on the recent characterization of actin genes of *B. ostreae*, specific primers were designed for real-time PCR using SYBR® Green chemistry. Specificity was demonstrated by the unique melting temperature peak observed in positive samples and by the lack of amplification in samples of oysters infected by closely related parasites, including *Bonamia exitiosa*. A calibration curve using a cloned template was defined to estimate copy number. The assay had a 6 log- dynamic range, mean inter- and intra-assay variation coefficients of <1% and a minimum detection limit of 50 gene copies per reaction. Using infected oyster samples as templates, the assay was at least 10-fold more sensitive than conventional PCR. The quantitative assay was applied to test 132 oysters, and results were compared with the heart imprint method. There was a strong correlation between both techniques, and the results showed that the real-time PCR assay should be useful for studies of the ecology of *B. ostreae* and its host–parasite relationship.

Keywords: *Bonamia ostreae*; Real-time PCR; Detection; Quantification; *Ostrea edulis*; Heart imprints

1. Introduction

Bonamia ostreae is an intracellular parasite of the flat oyster (*Ostrea edulis*), has a severe pathogenic effect on its host and causes major economic losses. This parasite is reported in Europe and North America [1, 2, 3, 4, 5, 6, 7, 8]. The closely related parasite, *Bonamia exitiosa*, was considered exotic to Europe and was only reported in the New Zealand dredge oyster (*Ostrea chilensis*) [9] and in the Australian mud oyster (*O. angasi*) [10, 11, 12] until its detection in Spain in the European flat oyster (*O. edulis*) in 2007 [13]. *B. exitiosa* was reported in *O. edulis*, sometimes in association with *B. ostreae* inside the same individuals without increased mortality. *B. ostreae* and *B. exitiosa* appear to be very similar in morphology using traditional diagnostic tools (including histology or tissue imprints) used for disease surveillance [13]). Molecular tools presently available, such those based on PCR and *in situ* hybridization [14, 15, 16], target the 18S ribosomal RNA gene which is conserved among species of *Bonamia* [17]. These detection tools do not allow the delineation of *Bonamia* species, such that DNA sequencing needs to be used for species identification and differentiation. In an epidemiological context, tools enabling specific and rapid diagnosis of *B. ostreae* are required.

A lag time of three to five months is usually reported before the parasite is detected in oysters in an area affected by *Bonamia* [18, 19, 20]. However this inability to detect the parasites is probably due to a lack of sensitivity of diagnostic tests used. The use of molecular tools, such as PCR, is proposed to increase the sensitivity of the diagnostic approach, as suggested previously by Lynch et al. [21]. Prespawing oysters can die from bonamiosis [22], but individuals of > 2 years of age appear to be more susceptible to the disease [18, 23, 24, 25], and death usually occurs concurrently with the highest intensity of infection [26, 27]. Therefore, quantitative data on parasite burdens will be valuable for disease investigations and stock management.

Bonamiosis due to *B. ostr*eae has an annual pattern that seems to differ according to the study site and climate or season. Two year-old oysters placed in Galicia showed maximum infection levels in August and September [28]. Maximum prevalence and infection levels in oysters sampled over a two years period in Ireland were recorded in spring and winter [25]. In The Netherlands, the peak of infection seems to occur in November-December [3, 19]. In France, prevalence also undergoes some fluctuation within a year, and is usually lowest in summer and highest in winter (Arzul, unpublished data).

This information raises issues regarding the sensitivity of diagnostic tests used in these studies and their suitability in studies aiming to estimate the prevalence and intensities of infection during an outbreak. Clearly, a robust, sensitive and quantitative assay is required to determine prevalence and intensity of infection. In the past, a semi quantitative assay based on examination of tissue imprints using oil immersion (x1000) under a light microscope has been used to estimate infection intensity [29]. Nevertheless, this technique is only semi-quantitative and skilled technicians are required to carry out the assay. Some real-time PCR assays targeting the 18S rDNA gene have been established for the detection of *Bonamia* species [8, 12], but were not quantitative

In this context, the objective of the present work was to develop a rapid and accurate technique allowing specific detection and quantification of *B. ostreae* in oysters. We developed a real-time PCR using primers targeting one of the actin genes characterized recently for *B. ostreae* [30], and we compared results with those achieved using heart imprint (HI) testing as well as conventional PCR [15].

2. Material and Methods

2.1. Oysters

Gill samples from flat oysters (*O. edulis*) were collected from various locations in France: Quiberon bay (South Brittany), Cancale (North Brittany), Granville oyster bed (Normandy) and La Tremblade (Charente Maritime). Samples of 5 mm were fixed in 90% ethanol. Gill pieces from oysters infected by parasites closely related to *B. ostreae* were also used to test the specificity of the primers and the PCR. These samples represented different oyster species which had been confirmed to be infected by *B. exitiosa* or related parasites; three *O. chilensis* originated from New Zealand, four *O. chilensis* from Chile, two *O. puelchana* from Argentina, one *O. angasi* from Australia, 27 *O. edulis* from Galician Rias, Mediterranean Sea and Adriatic sea. One *O. edulis* originating from The Netherlands, known to be infected with *Haplosporidium armoricanum*, and two *Crassostrea gigas* from Canada, known to be infected with *Mikrocytos mackini*, were also included the panel of control samples used to test the specificity of the assay.

2.2. Heart imprint testing, and semi-quantification

A heart imprint was performed on oyster that consisted of dissecting out the ventricle, drying it on paper and then performing several imprints on a slide. After air-drying for 5 min, imprints were fixed in absolute ethanol and then stained with Hemacolor® kit (Merck). Slides were observed under a light microscope (Olympus BH 51). The numbers of parasites were estimated on 3 to 5 microscopic fields of view (immersion oil X 100 objective, field diameter 220 µm, area 0.038 mm²) for each individual and the level of infection was determined by calculating the mean of these 3 to 5 examinations. The level of infection by the parasite classified as follows:
negative results (0), when no parasite was detected,
light infections (1+), when a mean of 10 or fewer parasites was detected per microscopic field of view
moderate infections (2+), when a mean between 10 and 50 parasites was obtained per field,
heavy infections (3+), when a mean of more than 50 parasites was obtained in each field.

2.3. DNA extraction

Gill tissue (25 mg) was collected from each oyster for DNA extraction using a QiaAmp DNA Mini-kit (Qiagen) according to the manufacturer's instructions. DNA was eluted and resuspended in 50 µL of sterile, deionised water and then diluted to a final concentration of 100 ng µL^{-1} for use in conventional PCR and 5 ng µL^{-1} in real-time PCR.

2.4. Conventional PCR conditions and parasite species confirmation by PCR-RFLP

Conventional PCR was performed according to Cochennec et al. [15]. The species of parasite was determined by digesting PCR products by *Bgl*I and *Hae*II for 1 h [31, 32]. The efficiency of this method (PCR-RFLP) was tested independently by sequencing 57 PCR products, revealing same digestion profiles as those of *B. ostreae*. PCR products were sequenced bidirectionally using the Big Dye V3 sequencing kit (Applied Biosystem, U.S.A.) and plasmidic primers (TopoF: 5'-GAC CAT GAT TAC GCC AAG C 3' and TopoR: 5'CCC AGT CAC GAC GTT G-3'). Sequences obtained were compared with those available in the GenBank database using BLAST algorithm [33]. Comparisons showed that the sequences of all of these PCR products corresponded to those representing *B. ostreae* (AF192759, AF262995, AF162087)

2.5. Design of primers specific for actin 1 gene of B. ostreae

Primers for the real-time PCR were designed based on the actin 1 gene sequences (GenBank accession nos. **AM410919**, **AM410920** and **AM410921**) determined by Lopez-Flores et al. [30]. The primers were: forward-Bo A1F (5'-GCT TCG ACC GAA AGT TCC G-3') and reverse-Bo A2R (5'-GGCGAAGAGGTCTTTTCTGA-3'). These primers amplified a 201 bp product in PCR.

2.6. Preparation of constructs as standards

For the quantification of the intensity of *B. ostreae* infection in oysters, the actin 1 gene was amplified by PCR, cloned and used as standard control to generate a standard curve. Briefly, PCR products were obtained by amplifying a fragment of the actin 1 gene using primers Bo A1F and Bo A2R from the total genomic DNA extracted from highly infected oyster. PCR products were then cloned using the TOPO TA cloning kit (Invitrogen), according to manufacturer's recommendations; positive clones were then selected for plasmid DNA purification by QIAFilter$^{®}$ Plasmid Midi Kit (Qiagen).

Plasmid DNA was linearized prior to being diluted for PCR for the determination of the standard curve. Linearization was performed by digesting plasmid DNA using *Xho* I for 1 h at 37 °C. The enzyme was then inactivated by incubation for 15 min at 65 °C. DNA concentration was determined using a spectrophotometer, and gene copy number was estimated using serial dilutions of cloned DNA; DNA concentration in g µL^{-1} x 6 10^{23} copies mol^{-1}/molecular weight of transformed plasmid in g mol^{-1}.

Plasmid DNA suspensions were then 10-fold serial diluted from 4 x 10^{6} copies µL^{-1} to 40 copies µL^{-1}. Two additional dilutions were used for actin gene copy estimation: 20 and 10 copies µL^{-1}. The PCR efficiency and correlation coefficient were estimated using the standard curve.

To examine the possibility that non target DNA would interfere with the assay, additional standard curves were developed by spiking 25 ng of DNA from non infected control flat oyster with a serial dilution of plasmid DNA. Moreover, to ensure that intact *B. ostreae* DNA behaved the same as plasmid DNA, additional standard curves were established for the PCR using genomic DNA extracted from gills of a known infected flat oyster and 10-fold serial diluted from 5 to 5 10^{-4} ng µL^{-1}. The PCR efficiency of dilution series of infected oyster extract, plasmid DNA in presence of oyster DNA was compared with that of plasmid DNA alone.

2.7. Real-time PCR conditions and quantification

Quantitative PCR was performed using a Mx3000 Thermocycler sequence detector (Stratagene). All reactions were carried out in 96-well plates in duplicate or triplicate in 25 µL comprising 5 µL of genomic DNA (sample) or plasmid DNA (positive control), 12.5 µL of Brilliant$^{®}$ SYBR$^{®}$ Green I PCR Master Mix (Stratagene), 2.5 µL of forward primer (2.4 µM), 2.5 µL of reverse primer (1.2 µM), 0.375 µL of Reference Dye (1mM) and 2.125 µL of distilled water. Each PCR run included negative controls consisting in reaction mixtures without DNA and specimens that were known to contain no *Bonamia ostreae* DNA.

The following cycling conditions were used: 95°C for 10 min, 40 cycles of amplification at 95°C for 30 s, 60°C for 1 min, 72°C for 30 s. The fluorescence signal was collected at the end of each annealing step (60°C). A post PCR melt curve was run according to the Mx3000 Thermocycler sequence detector User's Manual: a 30 s hold at 95°C, a 30 s hold at 60°C, and a 40 min slow ramp from 60 to 95°C. .

Threshold cycles (C_t), defined as the cycle at which a statistically significant increase in fluorescence output above background is detected, were calculated automatically by the Stratagene thermocycler software. C_t values were calculated as averages of the three replicates or duplicates within each reaction. Quantitative standards (serial dilution of plasmid DNA) and no template controls (NTC = deionised, sterile water) controls were included in each PCR run. The

estimation of the actin gene copy number for *B. ostreae* was carried out by comparing C_t values obtained for each sample with the standard curve.

2.8. Specificity of real-time PCR assay

The specificity of the amplified products was confirmed by a melting curve analysis. In addition, oysters detected positive by real-time PCR were tested by PCR-RFLP and sequencing in order to confirm the parasite species. DNA extracted from different species of oysters infected by parasites other than *B. ostreae* was subjected to real-time PCR to test specificity.

2.9. Sensitivity

The sensitivity of the SYBR® Green - based real-time PCR assay was measured using serially diluted plasmid DNA containing the actin 1 gene. The range consisted of 10-fold dilutions between 4×10^6 copies μL^{-1} and 4 copies μL^{-1}. The lowest dilution that showed specific amplification was further diluted (two-fold) serially. Genomic DNA extracted from two highly infected oysters was also diluted (10-fold) serially and subject to conventional PCR according to Cochennec et al. [15] as well as real-time PCR using the present real-time PCR assay. The lowest dilution that showed specific amplification was further diluted (two-fold) serially and tested by both assays.

2.10. Assay precision

Triplicate C_t values obtained from a dilution series (from 4×10^7 to 4×10^2 copies μL^{-1}) of plasmid preparation were used to measure intra assay coefficient of variation. Three separate dilution series (from 4×10^7 to 40 copies μL^{-1}) of plasmid were tested independently on three different days. The mean, standard deviation and variation coefficient were calculated separately for each plasmid dilution in order to evaluate inter-assay variation.

2.11. Statistical analyses

The C_t values, the deduced numbers of actin gene copies μg^{-1} of total DNA and the estimated numbers of parasites per tissue imprint were log(x+1)-transformed prior to statistical analyses, in order to improve the normality of the data and the homogeneity of variance [34]. Pearson's correlation coefficients between real-time PCR and heart imprint results were determined using the procedure PROC CORR from the SAS software package, and a linear regression was performed between the log-value of numbers of parasites per tissue imprint and the log-value of actin gene copies μg^{-1} of total DNA (estimated by real-time PCR) using the procedure PROC REG [35].

3. Results

3.1. Standard curve and dynamic range

Linear relationship between plasmid DNA copies and C_t values was observed for over 6 $\log_{10}$ dilutions (from 40 to 4 x 10^6 copies µL^{-1}) with a regression coefficient (r) of 0.99, a slope of – 3.345 (± 0.0222) suggesting an optimum PCR efficiency ([$10^{(-1/slope)}$ -1]) of 101.3% ± 2.53 (Table 2). The PCR efficiencies for plasmid DNA spiked with 25 ng not infected oyster DNA per reaction and for infected oyster DNA were similar to PCR efficiency obtained for plasmid DNA alone. In the presence of oyster DNA, the plasmid showed a correlation coefficient of 0.999 and PCR efficiency of 97.4%. The PCR efficiency for DNA extracted from *B. ostreae* infected oyster was 102.2 % with a correlation coefficient of 0.998.

3.2. Specificity of real-time PCR assay

A unique melting peak at 81.7°C ± 0.21 was observed after the real-time PCR with DNA extracted from oysters confirmed to be infected by parasites belonging to the genus *Bonamia* by heart imprints (Fig. 1). Eight of these samples were tested by PCR-RFLP and sequenced in order to confirm that these oysters were infected by *B. ostreae.* PCR products from two of the samples were also cloned and then 19 and 10 clones were subsequently tested by PCR-RFLP. All restriction profiles and sequences were the same as *B. ostreae* ones (AF192759, AF262995, AF162087).
The assay was applied to DNA extracted from 37 individual oysters infected by *B. exitiosa* or closely related parasites, one oyster known to be infected by *H. armoricanum* and two oysters infected by *M. mackini*. No amplification was detected for any of these samples. As expected, the NTC did not yield an amplicon.

3.3. Sensitivity of real-time PCR and comparison with conventional PCR

The sensitivity of the assay was evaluated by using serial dilutions of plasmid DNA (from 4 x 10^6 copies µL^{-1} to 4 copies µL^{-1}). The real-time PCR was able to achieve amplification from as few as 10 copies µL^{-1} (50 plasmid copies per reaction).
Two serial dilutions prepared from DNA extracted from highly infected oysters were tested twice by the real-time PCR assay and by conventional PCR. Quantification of *B. ostreae* actin gene copies was estimated at 5.64 10^5 copies ng^{-1} of DNA in sample 1 and at 1.61 10^5 copies ng^{-1} of DNA in sample 2 by our quantitative assay
The last dilution which allowed repeated detection of *B. ostreae* by real-time PCR was 5 x 10^{-4} ng µL^{-1} (corresponding to 1.41 x 10^3 parasite actin gene copies per reaction) for the first sample tested and 5 x 10^{-4} ng µL^{-1} (corresponding to 4.03 x 10^2 parasite actin gene copies per reaction) for the second sample tested. For the same amounts of DNA, conventional PCR showed repeated positive signal until dilution 5 x 10^{-1} ng µL^{-1} (estimated to correspond to 1.41 x 10^6 parasite actin gene copies per reaction by real-time PCR) for sample 1 and 5 x 10^{-3} ng µL^{-1} (estimated to correspond to 4.03 x 10^3 parasite actin gene copies per reaction by real-time PCR) for sample 2.

3.4. Assay Precision

The PCR assays performed on triplicate from a same plasmid DNA dilution on the same plate and on three independent plasmid DNA dilution series on different days demonstrated low variability (Tables 1 and 2). The mean intra-assay coefficient of variation calculated from triplicate C_t values was 0.69% ± 0.29. The coefficient of variation of replicate tests on different days was 0.71% ±0.48 %.

3.2. Testing of samples from oysters

Samples (n = 132) were tested conventionally *via* heart imprints and conventional PCR as well as real-time PCR. Heart imprints allowed categorizing samples according to their level of infection by *B. ostreae*; 43 samples were test negative using this approach; 24 samples had a light infection (with a mean of 1 to 6 parasites per microscopic field of view per oyster); 17 exhibited moderate infection level (with a mean of 10.4 to 44 parasites per microscopic field of view per oyster); 48 oysters had a high intensity of infection (with a mean of 54.6 to 309 parasites per microscopic field of view). Details of examination of heart imprints for oysters found infected are given in Tables 3 to 5.

Conventional PCR gave results that were very similar to those achieved using the heart imprint examination: all the oysters found to be test negative in the cytological technique were also test negative using the PCR . All oysters found infected, except one individual lightly infected by heart imprint, appeared positive by PCR.

The C_t values of real-time PCR demonstrated differences in the amount of parasite actin gene copies between oysters found not infected, lightly, moderately and highly infected (Fig. 2).

Real-time PCR assay conducted on oysters that were test negative by heart imprints and by conventional PCR did not yield any specific amplification for 24 samples. A specific amplification, as confirmed by the melting curve analysis, was obtained for both duplicates of 10 samples and for one of the duplicates of 9 samples. C_t values obtained for these 19 samples considered positive by our assay were above 34.2 and presented a median of 36.49. These Ct values were outside the range of our standard curve and thus did not allow us to estimate parasite actin gene copies.

Real-time PCR performed on the three groups of oysters that were test positive by heart imprint revealed mean of actin gene copies μg^{-1} of DNA ranging between 2.77 x 10^{-6} (±1.1 x 10^6) and 1.02 x 10^7 (±5.53 x 10^6) with corresponding Ct values of 29.86 and 23.34 respectively (Tables 3, 4 and 5).

The number of *B. ostreae* actin gene copies per oyster cell was deduced from the mean of DNA content of *O. edulis* genome estimated at 2.33 pg [36] and making the assumption that cells in tested samples corresponded to diploid, non-dividing cells. Oysters detected lightly, moderately and highly infected by heart imprints related to a mean of 6.44, 12 and 23.34 parasite actin gene copies per cell respectively.

Comparison of results obtained by real-time PCR and by heart imprint yielded a Pearson's correlation coefficient of – 0.90 between log (1+ number of parasites estimated by heart imprint) and log (1+ C_t value) ($p< 0.0001$) and 0.82 between log (1+ number of parasites estimated by heart imprint) and log (1+ deduced numbers of actin gene copies μg^{-1} of total DNA) ($p< 0.0001$) (Fig. 3).

4. Discussion

Bonamiosis due to *B. ostreae* is a systemic disease that causes mortality in affected stocks of *O. edulis*. Host mortality is correlated with a high intensity of infection, which can be estimated by tissue imprints according to the scale proposed by Hervio et al. [29]. However, this technique is not specific, is semi-quantitative and categorises oysters in four groups: not infected, lightly infected, moderately infected and highly infected. Here, we have developed a rapid, specific and sensitive real-time PCR assay allowing detection and quantification of *B. ostreae* DNA in oyster samples.

The diagnosis of mollusc diseases usually relies on histology and cytology (including tissue imprints). However, these techniques do not usually achieve specific identification or delineation, and molecular tools are generally required for species confirmation. Nevertheless, some tools available for the diagnosis of bonamiosis are not specific and produce an amplification product for all members of the genus *Bonamia* and closely related parasites within the Order Haplosporidia, such as *Haplosporidium nelsoni* [17]. In this case, confirmation of the parasite species is determined by sequencing the amplicon. In the past, geographical distribution and host specificity of some members of the genus *Bonamia* was distinct, with *B. ostreae* being reported in *O. edulis* in Europe and North America and *B. exitiosa* being described in *O. chilensis* in New Zealand and *O. angasi* in Australia. Based on this information, a consistent in morphology to *Bonamia* in flat oysters in Europe was considered to be *B. ostreae* but the specific identity had not been verified by molecular analysis.

The distribution and host specificity have been updated regularly, with detection of new *Bonamia* isolates in new host species in new parts of the world. More specifically, in October 2007, *B. exitiosa* was reported for the first time in flat oysters *O. edulis* from Galicia, Spain [13]. In this context, the development of specific tools for the rapid detection and discrimination of *Bonamia* parasites was required. Based on the recent characterization of actin genes of *B. ostreae* [30], we designed primers for a real-time PCR assay. Eight samples yielding amplification by this assay with an expected melting peak of 81.7°C ± 0.21 were tested by PCR-RFLP which confirmed that these oysters were infected by *B. ostreae*. Additionally, two samples were cloned, and 19 and 10 clones were tested by PCR-RFLP. The digestion profile for all samples was of the same as *B. ostreae* one suggesting that these two oysters were not co infected by other *Bonamia* species. The real-time PCR assay established was then applied to genomic DNA extracted from oysters infected by different members of the genus *Bonamia* as well as from oysters infected by *H. armoricanum* and *M. mackini*. The lack of amplification in all these samples shows a strong analytical specificity of the technique.

Quantification was achieved using a standard curve constructed by amplifying known amounts of target DNA in parallel set of reactions (ABI 2006). When a target DNA originates from an uncultivated micro-organism, plasmid DNA standards are often used. Such approach was already developed for detection and quantification of pathogens including protistans like *Plasmodium falciparum* [37], *Alexandrium minutum* [38] or *Babesia gibsoni* [39]. *B. ostreae* cannot be cultured *in vitro*; the development of standard curve using plasmid DNA containing the targeted parasite DNA region Bo A1F Bo A2R has allowed quantification of actin genes in tested samples over a 6 log range with an efficiency of 101.3%. Addition of non-target DNA as well as testing DNA extracted from infected oysters did not impair the efficiency of the reaction; 50 plasmid copies per reaction could be successfully detected by our real-time assay and was considered as the detection limit for this method. However, it is recognised that plasmid DNA is more easily amplified than mix of target and non-target DNA [38, 40]. Dilutions prepared from highly infected individuals were tested by real-time and conventional PCR according to Cochennec et al. [15] in order to compare detection limit of both assays. Although the 18S rDNA gene, targeted by the conventional PCR assay might be present in multiple copies in the

parasite genome, real-time PCR appears to be at least ten-times more sensitive than conventional PCR, depending samples tested.

Tests conducted to evaluate intra- and inter-assay variability revealed very low values (<1%, Tables 1 and 2), and good repeatability and reproducibility of results.

In the present study, we recorded results in copy numbers per μg of total DNA template. Recording results as copy numbers mg^{-1} of tissue might be problematic because of differences in DNA extraction efficiency, in effects of sample size and sample quality. However, number of *B. ostreae* actin gene copies per oyster cell was estimated by taking into account results of Rodriguez –Juiz et al. [36] and by making the assumption that all cells in the samples tested were diploid and not undergoing division.

The application of our assay to 132 oysters examined by heart imprints and categorised in 4 groups (not infected; lightly infected; moderately infected and highly infected) after observation of five microscopic fields of view per oyster served to validate the assay. All oysters detected shown by heart imprint or PCR to be test positive were also positive using the quantitative PCR assay. Besides, 19 oysters found not to be infected by heart imprint and conventional PCR yielded specific amplification using real-time PCR. C_t values obtained for these 19 samples were outside of the range of the standard curve and thus did not allow an estimation of actin gene copy number. These results suggest that samples shown to be test negative by heart imprint and conventional PCR could be lightly infected by *B. ostreae* and thus could be test positive y real-time PCR. The lack of sensitivity of the heart imprint approach is recognised as a limitation of assay, and molecular tools should have increased sensitivity. However, this is not always the case [41]. Moreover, molecular tools detect parasite DNA and do not confirm presence of viable pathogens and established infection. Interpretation of results for samples that are negative by heart imprints and positive by real-time PCR is thus difficult and open to debate.

Comparison of the results obtained by heart imprints and real-time PCR showed a very good correlation (0.82, *p*< 0.0001) between both techniques. Oysters shown to be lightly infected by heart imprint showed a mean of actin gene copies $μg^{-1}$ of total DNA of 2.77 x 10^6 or 6.44 actin gene copies per host cell. These results suggest that oysters with light infections by heart imprint already have advanced disease. Moreover, the lack of correlation between PCR and imprint observed in some studies most probably relates to an inability of the heart imprint method to detect low level primary primary infections.

In conclusion, a SYBR$^®$ Green-Based real-time PCR assay was established for monitoring and quantification of *B. ostreae* in the gill tissue of oysters. This assay was shown to be sensitive and specific and, moreover, was able to generate reproducible results. Comparison showed a very good correlation between heart imprint and the real-time PCR techniques. Thus this assay may be used in the future for detection and monitoring bonamiosis in various tissues of infected oyster and may be adapted for parasite detection in the environment to improve the knowledge of the parasite life cycle within and outside of the host.

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Tables

Copies	C_t value			Mean value	S.D.	CV	Intra-assay variability
	C1	C2	C3				
1.95e+008	13.81	13.46	13.71	13.66	0.15	1.08%	Mean
1.95e+007	16.90	16.65	16.77	16.77	0.10	0.61%	SD
1.95e+006	20.15	19.92	19.62	19.90	0.22	1.09%	
1.95e+005	23.72	23.48	23.64	23.61	0.10	0.42%	0.69%
1.95e+004	26.91	26.71	26.61	26.74	0.12	0.47%	0.29%
1.95e+003	30.33	30.10	30.01	30.15	0.13	0.45%	

Table 1 – Intra-assay variability calculated from triplicate (C1, C2 and C3) of a same plasmid DNA dilution on the same plate. SD: Standard Deviation. CV: Coefficient of Variation

Copies	Ct value (mean of triplicate)			Mean value	S.D.	CV	Inter-assay variability
	Expt. 1	Expt. 2	Expt. 3				
1.95e+008	13.66	13.66	13.62	13.65	0.02	0.12%	Mean
1.95e+007	16.77	16.97		16.87	0.10	0.58%	SD
1.95e+006	19.90	20.45	20.09	20.15	0.23	1.14%	
1.95e+005	23.61	24.24		23.93	0.31	1.31%	
1.95e+004	26.74	27.31	26.87	26.97	0.24	0.90%	0.71%
1.95e+003	30.15	30.29		30.22	0.07	0.23%	0.48%
1.95e+002			33.73	33.73	0.00	0.00%	

Inter assay data

	R^2	Slope	Efficiency
Exp1	0.99	-3.32	100.20
Exp2	0.99	-3.37	104.80
Exp3	1.00	-3.35	98.90
Mean	0.99	-3.35	101.30
SD	0.00	0.02	2.53

Table 2 – Inter-assay variability calculated from three independent plasmid DNA dilution series tested by real-time PCR on different days (Expt.1, Expt. 2 and Expt. 3). SD: Standard Deviation. CV: Coefficient of Variation. R : Correlation coefficient

	Number of parasites per microscopic field of view						Conventional PCR	Real-time PCR C_t value (mean of duplicates)	Calculated number of actin gene copies µg^{-1} of DNA	Estimated number of actin gene copies per oyster cell
	1	2	3	4	5	mean				
1	2	1	1	2	1	1.4	+	27.67	5.09E+05	1.18E+00
2	1	5	2	2	2	2.4	+	30.01	1.07E+05	2.49E-01
3	1	8	3	1	2	3	+	29.47	1.53E+05	3.56E-01
4	1	2	2	1	1	1.4	+	29.79	1.24E+05	2.88E-01
5	5	2	1	1	3	2.4	+	28.74	2.50E+05	5.81E-01
6	1	0	0	0	0	0.2	+	32.75	1.71E+04	3.98E-02
7	7	4	4	7	6	5.6	+	23.74	7.07E+06	1.64E+01
8	5	13	1	3	5	5.4	+	20.67	5.50E+07	1.28E+02
9	1	0	0	1	0	0.4	+	31.21	4.79E+04	1.11E-01
10	1	2	1	1	2	1.4	+	31.76	3.31E+04	7.70E-02
11	3	4	1	1	2	2.2	+	32.97	1.47E+04	3.43E-02
12	1	1	4	1	1	1.6	-	33.73	8.90E+03	2.07E-02
13	1	0	0	0	0	0.2	+	29.30	1.72E+05	3.99E-01
14	4	1	2	1	1	1.8	+	35.45	2.82E+03	6.56E-03
15	2	0	0	0	0	0.4	+	27.55	5.53E+05	1.29E+00
16	8	1	1	1	1	2.4	+	30.60	7.20E+04	1.68E-01
17	5	2	3	2	2	2.8	+	28.65	2.65E+05	6.17E-01
18	1	4	1	1	1	1.6	+	32.43	2.08E+04	4.85E-02
19	1	2	1	1	1	1.2	+	30.47	7.81E+04	1.82E-01
20	1	1	1	1	1	1	+	32.49	2.00E+04	4.65E-02
21	1	1	2	1	1	1.2	+	31.42	4.12E+04	9.58E-02
22	2	4	1	2	2	2.2	+	30.22	9.22E+04	2.14E-01
23	3	2	1	2	1	1.8	+	29.65	1.35E+05	3.14E-01
24	5	3	5	4	6	4.6	+	25.93	1.65E+06	3.83E+00
Mean						2.025		29.86	2.77E+06	6.44E+00

Table 3 – Comparison of results obtained by heart imprints (based on examination of 3 to 5 microscopic fields of view), conventional PCR and real-time PCR for flat oysters detected lightly infected by heart imprints. The estimated number of actin gene copies per oyster cell is deduced from the mean of DNA content of *Ostrea edulis* genome estimated at 2.33 pg [36].

	Number of parasites per microscopic field of view						Conventional PCR	Real-time PCR C_t value (mean of duplicates)	Calculated number of actin gene copies μg^{-1} of DNA	Estimated number of actin gene copies per oyster cell
	1	2	3	4	5	mean				
1	8	22	18	12	7	13.4	+	32.56	2.28E+04	5.30E-02
2	32	33	48	57	22	38.4	+	28.27	3.62E+05	8.43E-01
3	15	34	21	8	26	20.8	+	26.35	1.25E+06	2.92E+00
4	46	93	28	24	10	40.2	+	22.96	1.12E+07	2.60E+01
5	13	11	21	29	34	21.6	+	24.22	4.95E+06	1.15E+01
6	18	27	19	27	24	23	+	24.40	4.42E+06	1.03E+01
7	23	37	34	23	24	28.2	+	25.48	2.20E+06	5.12E+00
8	24	22	28	18	9	20.2	+	24.00	5.72E+06	1.33E+01
9	8	12	18	9	15	12.4	+	23.43	8.25E+06	1.92E+01
10	8	12	6	11	15	10.4	+	24.99	3.06E+06	7.13E+00
11	1	31	21	65	4	24.4	+	23.51	8.26E+06	1.92E+01
12	5	17	16	16	8	12.4	+	25.25	2.58E+06	6.00E+00
13	12	18	23	9	26	17.6	+	23.96	6.17E+06	1.44E+01
14	14	37	53	43	11	31.6	+	23.75	7.11E+06	1.65E+01
15	32	32	12	12	24	22.4	+	24.50	4.31E+06	1.00E+01
16	23	30	50	65	53	44	+	24.24	4.50E+06	1.05E+01
17	43	20	33	40	15	30	+	22.7	1.31E+07	3.05E+01
Mean						24.2		24.97	5.15E+06	1.20E+01

Table 4 – Comparison of results obtained by heart imprints (based on examination of 3 to 5 microscopic fields of view), conventional PCR and real-time PCR for flat oysters detected moderately infected by heart imprints. The estimated number of actin gene copies per oyster cell is deduced from the mean of DNA content of *Ostrea edulis* genome estimated at 2.33 pg [36].

	Number of parasites per microscopic field of view						Conventional PCR	Real-time PCR C_t value (mean of duplicates)	Calculated number of actin gene copies µg^{-1} of DNA	Estimated number of actin gene copies per oyster cell
	1	2	3	4	5	mean				
1	77	23	79	90	14	56.6	+	25.59	2.05E+06	4.77E+00
2	28	57	88	80	90	68.6	+	23.64	7.22E+06	1.68E+01
3	9	20	68	89	90	55.2	+	23.51	7.84E+06	1.82E+01
4	123	290	59	44	57	114.6	+	22.04	2.02E+07	4.70E+01
5	25	86	55	60	75	60.2	+	22.80	1.88E+07	4.38E+01
6	70	55	40	75	33	54.6	+	24.35	7.00E+06	1.63E+01
7	62	78	53	78	55	65.2	+	24.39	4.43E+06	1.03E+01
8	86	90	66	104		86.5	+	23.53	8.16E+06	1.90E+01
9	113	181	162	202	146	160.8	+	24.70	3.63E+06	8.44E+00
10	113	79	79	112	92	95	+	24.17	5.21E+06	1.21E+01
11	96	109	64	134	158	112.2	+	23.91	6.27E+06	1.46E+01
12	83	168	75	71	109	101	+	23.76	6.93E+06	1.61E+01
13	44	30	80	110	262	105	+	23.73	6.43E+06	1.49E+01
14	138	235	162	154	154	169	+	23.34	8.43E+06	1.96E+01
15	161	140	79	53	120	111	+	22.36	1.66E+07	3.87E+01
16	277	204	1216	187	196	198	+	23.87	5.82E+06	1.35E+01
17	112	150	99	87	131	116	+	22.86	1.18E+07	2.74E+01
18	86	70	141	115	97	102	+	24.37	4.56E+06	1.06E+01
19	49	26	74	66	82	59	+	24.64	3.77E+06	8.78E+00
20	115	79	149	112	146	120	+	23.47	7.72E+06	1.80E+01
21	144	121	177	140	139	144	+	24.02	5.25E+06	1.22E+01
22	133	231	119			161	+	22.93	1.12E+07	2.61E+01
23	323	172	291	225	231	248	+	23.03	1.05E+07	2.43E+01
24	227	258	299	198	198	236	+	23.34	8.41E+06	1.96E+01
25	168	138	98	149	98	130	+	23.21	1.02E+07	2.36E+01
26	266	187	208	219	267	229	+	22.70	1.31E+07	3.05E+01
27	112	111	97	86	53	92	+	25.06	2.55E+06	5.92E+00
28	103	187	90	164	154	140	+	22.88	1.16E+07	2.70E+01
29	119	196	176	192	130	163	+	22.27	1.77E+07	4.12E+01
30	252	230	219	201	251	231	+	22.95	1.11E+07	2.57E+01
31	363	353	206	234	282	288	+	22.60	1.41E+07	3.28E+01
32	83	56	150			96	+	21.71	2.61E+07	6.08E+01
33	110	308	287	260	428	279	+	22.68	1.33E+07	3.09E+01
34	272	237	183	252	125	214	+	22.24	1.81E+07	4.21E+01
35	68	95	191	137	206	139	+	23.26	8.91E+06	2.07E+01
36	132	152	149	140	184	151	+	23.30	8.68E+06	2.02E+01
37	279	284	139	237	151	218	+	22.66	1.35E+07	3.13E+01
38	271	204	409	366	295	309	+	22.33	1.71E+07	3.97E+01
39	245	318	342	183	310	280	+	23.29	8.70E+06	2.02E+01
40	58	153	142	63	99	103	+	22.30	1.74E+07	4.04E+01
41	168	150	286	337	255	239	+	23.11	9.91E+06	2.30E+01
42	312	313	331	332	138	285	+	23.52	7.46E+06	1.73E+01
43	202	314	275	147	282	244	+	23.11	9.88E+06	2.30E+01
44	307	339	257	204	222	266	+	23.22	9.14E+06	2.13E+01
45	189	220	166	185	99	172	+	23.40	8.11E+06	1.89E+01

46	156	193	58	119	93	124	+		21.76	2.53E+07	5.88E+01
47	95	137	140	220	82	135	+		24.91	2.83E+06	6.58E+00
48	189	86	25	57	56	83	+		23.45	7.80E+06	1.81E+01
Mean						154			23.34	1.02E+07	2.38E+01

Table 5 - Comparison of results obtained by heart imprints (based on examination of 3 to 5 microscopic fields of view), conventional PCR and real-time PCR for flat oysters detected highly infected by heart imprints. The estimated number of actin gene copies per oyster cell is deduced from the mean of DNA content of *Ostrea edulis* genome estimated at 2.33 pg [36].
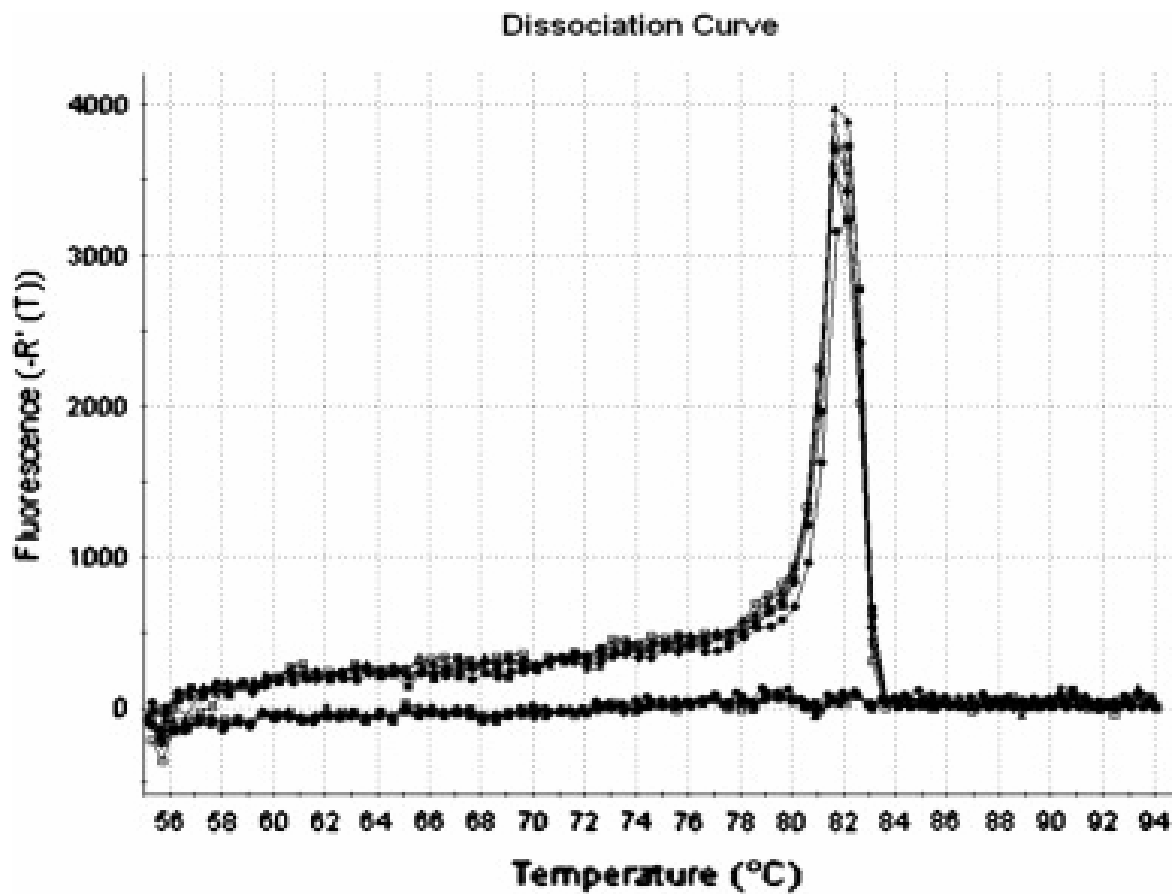
Figures

Fig. 1 – Melting curves of the amplified fragments generated by real-time PCR with DNA extracted from oysters confirmed to be infected by *Bonamia ostreae* (showing a melting peak between 81 and 82°C) and with DNA extracted non infected flat oysters (baselines).
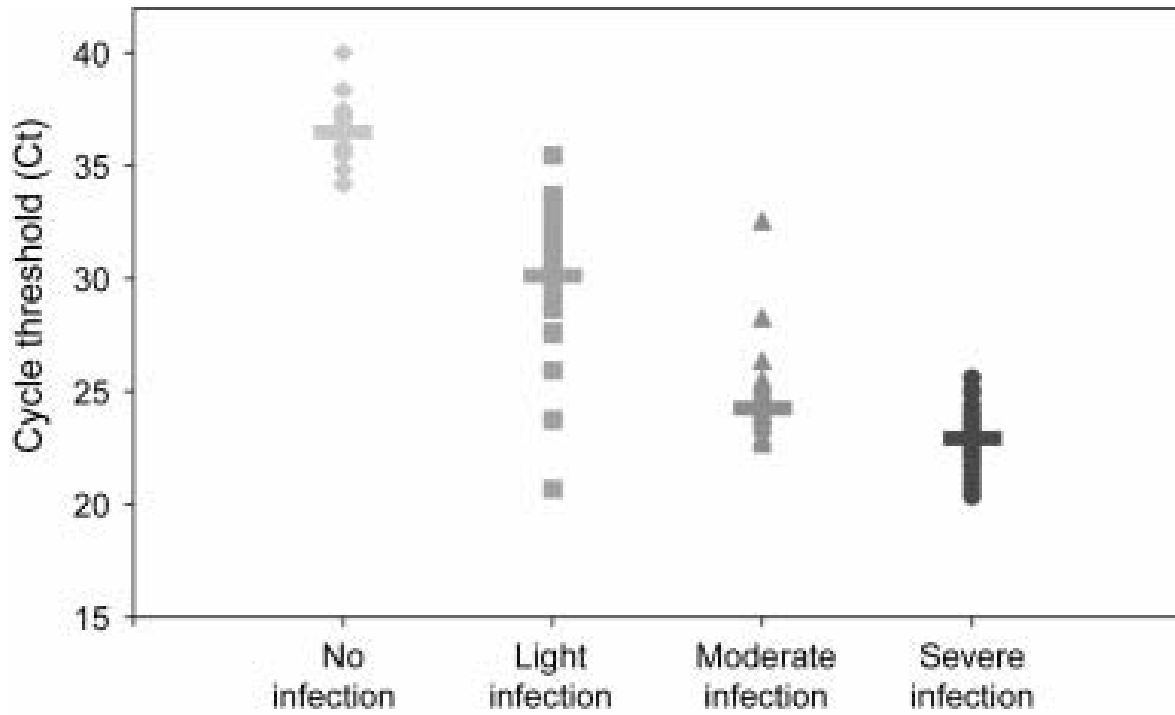
Fig. 2 – C_t values obtained by the real-time PCR assay in flat oysters found not infected, lightly, moderately and highly infected by heart imprints. The values are means of duplicate amplifications. Bars indicate median value.
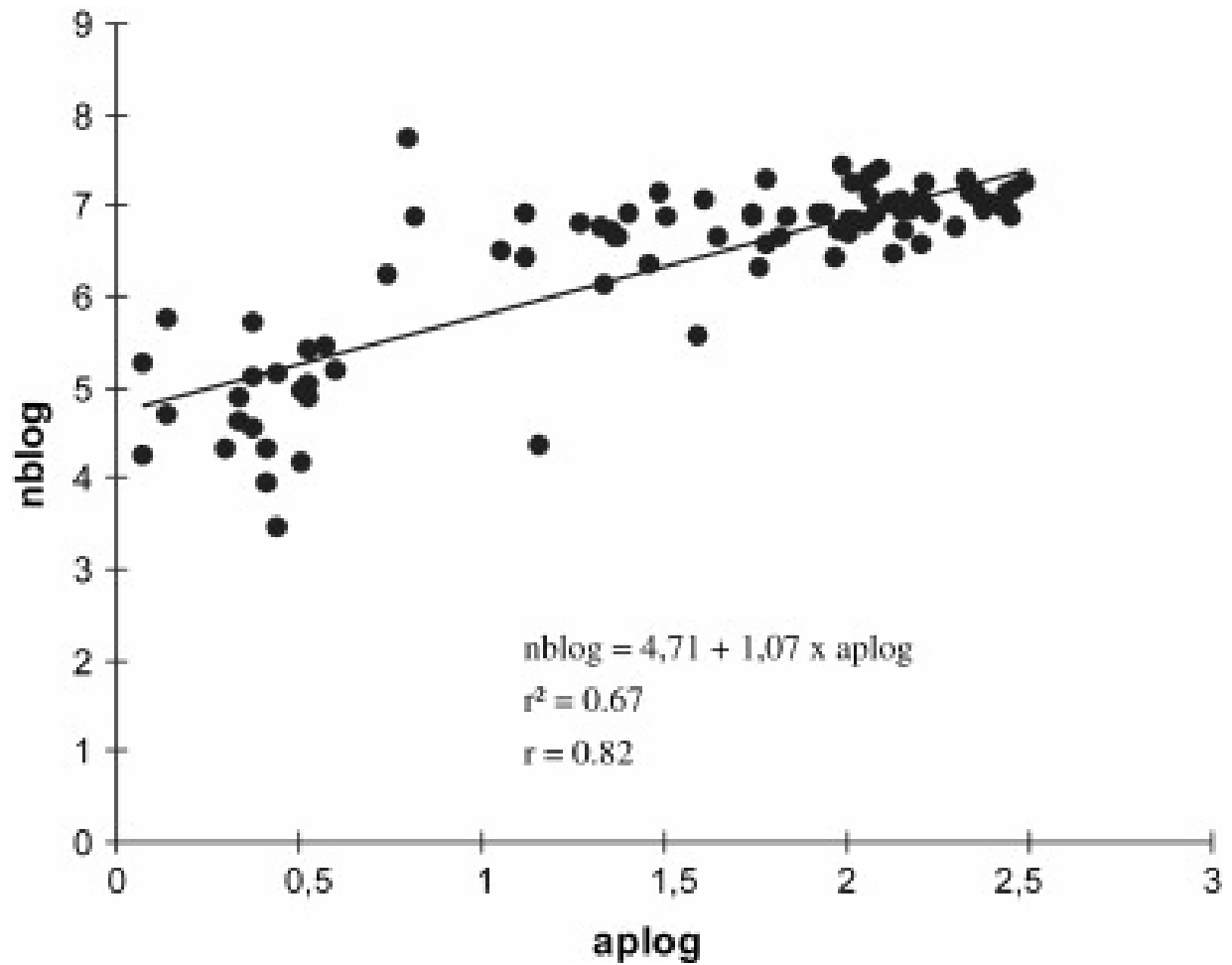
Fig. 3 - Linear regression obtained from log (1+ deduced number of *Bonamia ostreae* µg[-1] of total DNA) = nblog and log (1+number of parasites per microscopic field of view by examination of heart imprints) = aplog for 89 flat oysters tested in the present study (negative results for tissue imprints were excluded).