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Interlaboratory variability in screening for *Bonamia ostreae*, a protistan parasite of the European flat oyster *Ostrea edulis*

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ABSTRACT: The spread of the protozoan parasite *Bonamia ostreae* is of major concern to the European flat oyster *Ostrea edulis* industry. Many studies have looked at the sensitivity of individual methods available to screen for *B. ostreae,* but in this study, 3 separate laboratories examined 4 methods of diagnosis currently used routinely in laboratories: heart imprints, histology, polymerase chain reaction (PCR) and *in situ* hybridisation (ISH). The results were compared to estimate interlaboratory variability. Heart imprints and histology had the highest reproducibility amongst the 3 laboratories, with greatest agreement between detection of infected and uninfected individuals. PCR had the highest detection level in every laboratory. These positives were related to the presence of confirmed infections but also in unconfirmed infections, possibly due to the presence of traces of *B. ostreae* DNA in oysters where clinical infections were not observed. PCR, in combination with histology or ISH, provided the most reliable detection levels in every laboratory. Variation in results for PCR and ISH observed between laboratories may be due to the different protocols used by each laboratory for both methods. Overall, the findings from the 3 laboratories indicated that at least 2 methods, with fixed protocols, should be used for the accurate detection and determination of infection prevalence within a sample. This combination of methods would allow for a clearer and more precise diagnosis of *B. ostreae*, preventing further spread of the disease and providing more accurate detection levels and epidemiological information.

KEY WORDS: Bivalve · Protozoa · Diagnostics · Methodology · PCR · *In situ* hybridisation · Heart imprints · Histology

INTRODUCTION

The sustainability of the European flat oyster *Ostrea edulis* industry depends on the development of rapid and accurate diagnostic methods for monitoring the protozoan parasite *Bonamia ostreae*, and the more recently discovered *B. exitiosa*, to prevent the transfer of infected stock. It is essential to limit the transfer of these pathogens, because once they are introduced into a population, eradication is impossible (Van Banning 1987). At present, the main foundation of disease control by the World Organisation for Animal Health (OIE) is certification of stock as *B. ostreae*-free prior to movement. The diagnostic techniques recommended by the OIE are the screening of tissue imprints, the histological examination of tissue sections and genetic-based techniques such as polymerase chain reaction (PCR), restriction frag-

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ment length polymorphism (RFLP) and *in situ* hybridisation (ISH) (OIE 2012). Screening techniques should be selected primarily based on reliability and sensitivity, although ease of use, safety and cost should also be considered (Culloty & Mulcahy 2007).

The production of a heart imprint allows screening of the parasite in blood cells and determination of the intensity of infection. Heart imprints are economical and rapid to make in comparison to the other techniques commonly used (Zabaleta & Barber 1996, O'Neill et al. 1998, Carnegie et al. 2000, Culloty et al. 2003, Culloty & Mulcahy 2007, Lallias & Arzul 2008). The major disadvantages to the use of heart imprints is that *Bonamia ostreae*, due to its small size (2–5 µm), may go undetected when the infection is light or in its latent period (Carnegie et al. 2000, Cochennec-Laureau et al. 2003, Culloty et al. 2003, Da Silva & Villalba 2004, Lynch et al. 2008, Bower 2011) and that *B. ostreae* may be confused with other morphologically similar parasites, such as *Mikrocytos mackini* and *B. exitiosa* (Marty et al. 2006, Narcisi et al. 2010).

The main advantage of histology over heart imprints is the ability to make observations on the overall health and physiological status of the oyster (Diggles et al. 2003, Balseiro et al. 2006, Culloty & Mulcahy 2007, Lynch et al. 2008). The primary disadvantages of histology are low sensitivity, misidentification of certain pathogens, difficulty in screening larvae and spat and the length of preparation time (Cochennec et al. 1992, Carnegie et al. 2000, 2003, Diggles et al. 2003, Balseiro et al. 2006, Marty et al. 2006).

Standard PCR assay appears to be a more sensitive method of detection than the more traditional, cytological-based methods, detecting all classes of infection (Carnegie et al. 2000, Carnegie & Cochennec-Laureau 2004, Balseiro et al. 2006). It can also be used to detect the presence of *Bonamia ostreae* DNA prior to clinical manifestations of the disease and to screen spat and juvenile oysters (Lynch et al. 2005, Marty et al. 2006). DNA extraction and PCR is fairly rapid, taking 1 to 2 d to complete. RFLP analysis can be used to identify morphologically similar haplosporidians (Hine et al. 2001, Marty et al. 2006). RFLP is currently considered one of the most cost effective molecular markers available (Schlötterer 2004); however, the occurrence of false positives and false negatives is a major issue with PCR (Burreson 2000, Walker & Subasinghe 2000). Unlike histology and cytology, which possess semi-quantitative scales, standard PCR has no form of quantification (Diggles et al. 2003, Lallias & Arzul 2008) and thus necessitates the use of real-time PCR, a more expensive and complex method.

For the process of ISH, a digoxigenin-labelled probe is used to localise a *Bonamia ostreae*-specific DNA sequence in a section of oyster tissue. This allows an observer to visualise the parasite and to determine its exact location within the oyster (Culloty & Mulcahy 2007). In theory, ISH can be used in the identification of more cryptic stages of *B. ostreae* and for other potential carriers of the parasite (Carnegie et al. 2003, Culloty & Mulcahy 2007, Lynch et al. 2008, 2010). The crucial drawback to the use of ISH is the lack of specificity relating to the OIE mandated probe used in the screening of *B. ostreae*. The probe, as with PCR, currently uses Bo + Boas primers. In testing of this method in regions with multiple parasitic infections, the probe also attached to *Haplosporidium nelsoni* and *Bonamia exitiosa* (Carnegie & Cochennec-Laureau 2004, Bower 2011). However, in Ireland, where sampling for this study took place, *B. exitiosa* has not been detected to date. *H. nelsoni* has been detected in *Crassostrea gigas* and a single isolated case of *Ostrea edulis* but not in the regions sampled for this study (Lynch et al. 2013).

Although many individual studies have been carried out to assess the sensitivity of each method, few studies have carried out interlaboratory comparisons between methods and samples. In this study, 3 laboratories with long-term experience of screening for *Bonamia ostreae* carried out an interlaboratory calibration and assessment to evaluate the reproducibility of 4 methods for the detection of *B. ostreae* and to compare the results when using the same samples of oysters.

MATERIALS AND METHODS

A sample of *Ostrea edulis,* 60 animals in total, was collected from 2 *Bonamia ostreae*-endemic locations in Ireland, Clew Bay and Lough Foyle, in October 2010 and January 2011, respectively. Final analysis amongst all laboratories resulted in 30 of the 60 animals being screened by all 4 methods: 17 samples from Clew Bay and 13 samples from Lough Foyle. Three replicates of heart imprints and adjacent tissue sections for histology and ISH were prepared at the University College Cork (UCC, Cork, Ireland) for each individual oyster to be subsequently screened by the UCC, the Centro de Investigaciones Marinas (CIMA, Vilanova de Arousa, Spain) and the Centre for Environment, Fisheries and Aquaculture Science (Cefas, Weymouth, UK) laboratories. Three small pieces of gill tissue (approximately 5 mm) were also removed from each oyster at UCC to be screened

using PCR by each laboratory. All samples were coded to allow for blind testing.

Prevalence of infection using ventricular heart imprints

Each oyster was opened, the complete heart was removed and placed onto tissue paper, and the ventricular heart was imprinted onto 3 slides (1 slide for each laboratory). The imprints were air dried before being fixed in methanol for 2 min and stained with Hemacolour 2 and 3 (Merck) (Culloty et al. 1999). The imprints were examined in all laboratories under light microscopy at 400× and 1000× magnifications.

Processing and examination of histological sections

For histopathology, an oblique transverse section, consisting of mantle, gills, gonad and digestive gland, was taken from each animal and fixed in Davidson's fixative for 48 h (Shaw & Battle 1957, Howard et al. 2004). These tissue samples were then embedded in paraffin and cut into 5 µm sections. Finally, sections were placed on slides (3 slides, 1 for each laboratory) and stained with haematoxylin and eosin. The sections were examined under light microscopy at 400× and 1000× magnifications. Results were categorised as either positive or negative for the presence of *Bonamia ostreae*.

DNA isolation and PCR analysis

Three 5 mm pieces (1 piece for each laboratory) of gill tissue were collected from each animal and fixed in 95% ethanol in coded vials, which were then distributed to each laboratory. Different extraction protocols were used in each laboratory, as routinely occurs. At UCC, DNA was extracted from the tissue using the Chelex-100 method (Walsh et al. 1991, Lynch et al. 2010). At CIMA, the commercial Wizard Genomic DNA Purification Kit (Promega) was used, according to the manufacturer's protocol. At Cefas, the DNA was extracted from the tissue using the EZ-1 DNA tissue mini kit and an EZ-1 BioRobot (Qiagen), according to the manufacturer's protocol. The extractions were stored at −20°C. In all 3 laboratories, PCR analyses were performed with primers Bo + Boas, which amplified 300 bp from the small subunit ribosomal RNA (SSU rRNA) gene, using the method of Cochennec et al. (2000). All PCR assays included positive controls (*Bonamia ostreae* DNA) and a negative control (double-distilled water). The PCR products were run on agarose gel in buffer stained with ethidium bromide. All results were categorised as either positive or negative.

In situ hybridisation

Tissue sections were placed on silane-prep slides (Sigma Aldrich™), coded and distributed to each laboratory. In each laboratory, a probe was generated by PCR using primers Bo + Boas, which target the SSU rDNA gene, and incorporation of digoxigenin occurred during amplification of the 300 bp product (Cochennec et al. 2000). At UCC, all solutions and buffers used were made up according to the protocol described by Sambrook & Russell (2001) and Lynch et al. (2008) and cover slipped with DPX mountant (Sigma-Aldrich™). In CIMA, sections were prepared using a similar method to UCC and cover slipped with Histomount (Zymed, Invitrogen).

In Cefas, following preparation of the probe the sections were de-waxed by 2 immersions in Clearene (Leica Biosystems) and then 2 baths of 99% industrial methylated spirits (IMS). Sections were treated with Proteinase K in Tris/NaCl Buffer (TB) and incubated in a moist chamber for 15 min before dehydration in 99% IMS. Sections were fitted with Gene Frames (Anachem), overlaid with hybridisation buffer containing the digoxigenin-labelled probe and then sealed before being placed in a thermocycler to denature the probe. Sections were cooled on ice before an overnight incubation at 42°C. After Gene Frame removal, sections were washed twice in 1× saline sodium citrate, 6 M urea, 0.2% bovine serum albumin in distilled water at 42°C. Sections were blocked with 6% skimmed milk powder (Fluka, Sigma-Aldrich™) in TB at room temperature and then incubated with anti-Dig conjugated to alkaline phosphatase, diluted 1/300 in TB. Sections were placed in 5 successive washes of 1× TB followed by a brief immersion in 100 mM Tris-HCl. Nitro blue tetrazolium/bromo-chloro-indolyl phosphate in alkaline phosphatase buffer was added to the tissue and sections were incubated in the dark before immersion in 100 mM Tris-HCl. Finally, sections were placed in Nuclear Fast Red solution, rinsed in tap water and dehydrated in 99% IMS before being held in Clearene and cover slipped with Eukitt (Sigma-Aldrich™). All sections were examined under light microscopy at 400× and 1000× magnifications. Results were categorised as either positive or negative.

All 4 methods of screening were used to determine the percentage of oysters with positive parasite detection within each laboratory. The sets of results from all 3 laboratories were then compiled and compared.

RESULTS

Percentage of positive samples detected by each method

Heart imprints, histology and ISH were used to determine the prevalence of clinical infection following observation of the parasite. Heart imprints had the least variation amongst the laboratories, 30.0 to 36.7% prevalence of infection, while with histology and ISH, prevalence of infection varied from 30 to 40% and from 26.7 to 36.7%, respectively. PCR, detecting *Bonamia ostreae* DNA, had the highest variation in detection, 46.7 to 60% (Tables 1 & 2). Some positive results were obtained with this method in individuals that did not display clinical infection.

Reproducibility of results for each method

Heart imprint screening had the highest level of reproducibility amongst the 3 laboratories. Overall, 80% of the results, both positive and negative, from all 3 laboratories, agreed. Histology followed closely with an agreement of 77%. ISH had an agreement of 63.3%. The least overall agreement was observed using PCR, with only 53.3% of the sample results being the same amongst the 3 laboratories (Tables 1 & 3).

Detection rate with combined diagnostic methods

A combination of methods by pairs increased the percentage of positive cases. In the UCC laboratory, the combination of PCR with any of the other methods had the highest overall detection of *Bonamia ostreae* (63.3%). The combination of PCR + histology and PCR + ISH had the highest overall detection of *B. ostreae* (53.3%) at CIMA followed by heart imprints

Table 1. *Ostrea edulis* infected with *Bonamia ostreae.* Oysters in which *B. ostreae* was detected using each diagnostic method in each of the 3 laboratories (UCC: University College Cork, Ireland; CIMA: Centro de Investigaciones Marinas, Spain; Cefas: Centre for Environment, Fisheries and Aquaculture Science, UK). ISH: *in situ* hybridisation

Oyster no.	Heart imprints			Histology			PCR			ISH		
	UCC	CIMA	Cefas	UCC	CIMA	Cefas	UCC	CIMA	Cefas	UCC	CIMA	Cefas
1	–	–	–	–	–	–	+	+	+	+	–	–
2	+	+	+	+	+	+	–	+	+	+	+	+
3	+	+	+	+	+	+	–	+	+	+	+	+
4	+	+	–	+	+	–	–	+	+	+	–	–
5	+	+	–	+	+	–	–	+	+	+	–	–
6	+	+	+	+	+	+	–	+	+	–	+	+
7	+	+	+	+	+	+	+	+	+	–	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+
10	–	–	–	–	–	–	–	+	–	+	–	–
11	–	–	–	–	–	–	+	–	+	–	–	–
12	–	–	–	–	–	–	–	–	+	–	–	–
13	–	+	–	–	+	+	+	+	+	–	–	+
14	+	–	+	–	+	+	+	+	+	+	+	+
15	–	–	–	–	–	–	+	–	–	–	–	–
16	–	–	–	–	–	+	+	–	–	–	+	–
17	–	–	–	–	–	–	–	–	–	–	–	–
18	–	–	–	–	–	–	–	–	–	–	–	–
19	–	–	–	–	+	+	–	–	–	–	–	+
20	–	–	–	–	–	–	–	–	+	–	–	–
21	–	–	+	–	–	–	+	–	–	–	–	–
22	–	–	–	–	–	–	–	+	+	–	–	–
23	–	+	–	–	–	+	+	+	+	+	–	+
24	–	–	–	–	–	–	–	–	–	–	–	–
25	–	–	–	–	–	–	–	–	–	–	–	–
26	–	–	–	–	–	–	–	–	–	–	–	–
27	–	–	–	–	–	–	–	–	–	–	–	–
28	–	–	–	–	–	–	+	–	+	–	–	–
29	–	–	–	–	–	–	+	–	–	–	–	–
30	+	+	+	+	+	+	+	+	+	+	–	–

Table 2. *Ostrea edulis* infected with *Bonamia ostreae*. Percentage of oysters in which *B. ostreae* was detected using each diagnostic method in each of the 3 laboratories (summary of data in Table 1; abbreviations as in Table 1)

Laboratory	Heart imprints	Histology	PCR	ISH
UCC	33.3 (10/30)	30 (9/30)	46.7 (14/30)	36.7 (11/30)
CIMA	36.7 (11/30)	40 (12/30)	50 (15/30)	26.7 (8/30)
Cefas	30.0 (9/30)	40 (12/30)	60 (18/30)	33.3 (10/30)

Table 3. *Ostrea edulis*. Agreement (%) of results for all oysters, both positive and negative diagnosis, among the 3 laboratories (abbreviations as in Table 1)

Laboratory	Heart imprints	Histology	PCR	ISH
UCC vs. CIMA	90 (27/30)	90 (27/30)	60 (18/30)	70 (21/30)
UCC vs. Cefas	90 (27/30)	77 (23/30)	63.3 (19/30)	70 (21/30)
CIMA vs. Cefas	80 (24/30)	87 (26/30)	83.3 (25/30)	93.3 (28/30)
UCC vs. CIMA vs. Cefas	80 (24/30)	77 (23/30)	53.3 (16/30)	63.3 (19/30)

+ PCR (50%). The combination of PCR + histology had the highest overall detection of *B. ostreae* (66.7%) at Cefas followed by PCR + ISH (63.3%) and heart imprints + PCR (63.3%). Heart imprints + histology had the lowest overall detection of *B. ostreae* in all 3 laboratories (33.3–43.3%; Table 4).

Agreement in positive cases between diagnostic methods in each laboratory

A combination of heart imprints + histology screening had the highest percentage of infected individuals in common at UCC (90%) and CIMA (83.3%) while a combination of histology + ISH had the highest percentage in Cefas (83.3%). Histology + PCR at UCC had the lowest agreement (21.1%). At CIMA, PCR + ISH had the lowest agreement (44%) while at Cefas, heart imprints + PCR had the lowest agreement, 42.1% (Table 5).

DISCUSSION

In the samples screened, the overall prevalence of infection was approximately 33%. Most consistency occurred amongst laboratories with heart imprints, and the most variability with PCR. To assess clinical infections, heart imprints and histology gave good reproducibility amongst the laboratories, indicating that, apart from very low levels of infection (i.e. where just a few *Bonamia ostreae* cells are present), screening using these methods gave fairly consistent results. Light infections may have been overlooked due to the small size and focal nature of *B. ostreae* and the subjective nature of microscopic methods wherein just an imprint or a single transverse section, several microns thick, is screened (Culloty et al. 2003, Da Silva & Villalba 2004, Lynch et al. 2008). Also, the overall success of these methods is highly dependent on the fixation of the tissue and the preparation of the

Table 4. *Ostrea edulis* infected with *Bonamia ostreae*. Total/overall percentage of oysters in which *B. ostreae* was detected using a combination of diagnostic methods by pairs at UCC, CIMA and Cefas (abbreviations as in Table 1). The highest percentage detected at each laboratory is shown in **bold**

Laboratory	Heart imprints + Histology	Heart imprints + PCR	PCR + Histology	Heart imprints + ISH	PCR + ISH	Histology + ISH
UCC	33.3 (10/30)	**63.3 (19/30)**	**63.3 (19/30)**	43.3 (13/30)	**63.3 (19/30)**	43.3 (13/30)
CIMA	40.0 (12/30)	50.0 (15/30)	**53.3 (16/30)**	43.3 (13/30)	**53.3 (16/30)**	43.3 (13/30)
Cefas	43.3 (13/30)	63.3 (19/30)	**66.7 (20/30)**	40.0 (12/30)	63.3 (19/30)	40.0 (12/30)

Table 5. *Ostrea edulis* infected with *Bonamia ostreae*. Comparison of the agreement between methods for *B. ostreae*-positive (%) oysters at each laboratory (abbreviations as in Table 1)

	Heart imprints			PCR			Histology		
	UCC	CIMA	Cefas	UCC	CIMA	Cefas	UCC	CIMA	Cefas
PCR	26.3 (5/19)	73.3 (11/15)	42.1 (8/19)						
Histology	90.0 (9/10)	83.3 (10/12)	61.5 (8/13)	21.1 (4/19)	63.0 (10/16)	50.0 (10/20)			
ISH	61.5 (8/13)	46.2 (6/13)	58.3 (7/12)	53.8 (7/13)	44.0 (7/16)	47.4 (9/19)	31.6 (6/19)	46.2 (6/13)	83.3 (10/12)

slides, thus these ungovernable technical issues may have occurred with some of the slides (Diggles et al. 2003, Lynch et al. 2008).

Overall, PCR gave the highest rate of positive detection for *Bonamia ostreae* DNA in all 3 laboratories but also had the lowest agreement amongst laboratories. This was to be expected, as many studies have found that PCR produces a higher number of positives than light microscopy (Diggles et al. 2003, Lynch et al. 2005, 2008, Balseiro et al. 2006, Culloty & Mulcahy 2007). However, light *Bonamia ostreae* infections might give variable results amongst laboratories. This might be particularly true in our study, where different individual pieces of tissue were screened by PCR at each laboratory. Low infection levels and low numbers of *B. ostreae* cells, localised in particular host cells or tissues, might give variable results depending on whether infected or uninfected pieces of tissue were screened.

The high rate of detection associated with PCR in this study does not necessarily imply that PCR is the most effective detection method. Due to the design of the procedure, the pathogen cannot be visualised, and thus a viable pathogen and/or infection may not be present. The PCR may be detecting traces of *Bonamia ostreae* DNA (Burreson 2000). In contrast, since gill tissue was used in this study, PCR may also be detecting early infections which have not yet become systemic.

At present, the OIE recommends that histology or tissue imprints are used for surveillance, and when mortalities occur, PCR can be used in addition to these techniques (OIE 2012). Overall the findings of our study indicate that at least 2 methods should be used at all times for the accurate detection of prevalence of infection within a sample: a microscopy-based technique for visualisation of the parasite, the infection and associated pathology, and a molecular-based technique. Da Silva & Villalba (2004) concluded that every technique gives rise to false negatives, thus using several methods would lower the risk of misdiagnosing each individual. Lynch et al. (2008) found that the use of any 2 methods increased the likelihood of detecting *Bonamia ostreae.* Of the traditional microscopic methods, heart imprints appear to be the best choice, as this technique had the highest agreement of infected individuals amongst the 3 laboratories, and it was the most effective method at detecting light infections. Heart imprints are also fast to prepare (Diggles et al. 2003), an important factor when screening animals prior to movement. PCR may be considered the best molecular method of detection because although ISH has the ability to visualise the parasite, detection of light infections was greater with PCR. Also factoring in other aspects such as cost and preparation time, ISH is expensive and time consuming to undertake (Diggles et al. 2003, Lynch et al. 2008).

Diggles et al. (2003) recommended the refinement of heart imprints, using a method similar to the dot–blot methods used by Lightner & Redman (1998), in which haemolymph is hybridised with a digoxigenin-labelled probe. The combination of a quick and easy traditional method with a modern molecular technique may possibly lead to a sole, reliable diagnostic test for *Bonamia ostreae.*

The variation in the results amongst the 3 laboratories in the methods PCR and ISH, both complex and lengthy molecular tests with the possibility for highly varied methodologies, may be due to the different protocols used by each laboratory for both methods. Heart imprints and histology, both straightforward and concise methods, showed very little variation amongst the laboratories. These findings may imply the need for a standardised, fixed protocol for each molecular-based method to allow for optimisation of each assay and consistency in findings throughout all laboratories in the screening for *Bonamia ostreae.* The fact that the gill pieces used for PCR diagnosis were not the same for the 3 laboratories could also contribute to increased variability in the results obtained by this method, especially in light infections.

Although the EU strictly regulates the movement of *Ostrea edulis*, the fact that no diagnostic method is 100% accurate is of great concern. This study indicates that, at present, the use of both a microscopy-based method, to allow for visualisation of the parasite, and a molecular method, to increase sensitivity in low infections, would allow for a more precise diagnosis of *Bonamia ostreae* and that particular caution is required when screening light infections.

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LITERATURE CITED

Balseiro P, Conchas RF, Montes J, Gómez-León J, Novoa B, Figueras A (2006) Comparison of diagnosis techniques for the protozoan parasite *Bonamia ostreae* in flat oyster *Ostrea edulis.* Aquaculture 261:1135–1143

Bower SM (2011) Synopsis of infectious diseases and parasites of commercially exploited shellfish: *Bonamia ostreae* of oysters. Available at www.pac.dfo-mpo.gc.ca/science/species-especes/shellfish-coquillages/diseases-maladies/pages/bonostoy-eng.htm (accessed 4 February 2013)

Burreson E (2000) Disease diagnosis by PCR: foolproof or foolhardy? J Shellfish Res 19:642

Carnegie RB, Cochennec-Laureau N (2004) Microcell parasites of oysters: recent insights and future trends. Aquat Living Resour 17:519–528

Carnegie RB, Barber BJ, Culloty SC, Figueras AJ, Distel DL (2000) Development of a PCR assay for detection of the oyster pathogen *Bonamia ostreae* and support for its inclusion in the Haplosporidia. Dis Aquat Org 42:199–206

Carnegie RB, Barber BJ, Distel DL (2003) Detection of the oyster parasite *Bonamia ostreae* by fluorescent *in situ* hybridization. Dis Aquat Org 55:247–252

Cochennec N, Hervio D, Panatier B, Boulo V and others (1992) A direct monoclonal antibody sandwich immunoassay for detection of *Bonamia ostreae* (Ascetospora) in hemolymph samples of the flat oyster *Ostrea edulis* (Mollusca). Dis Aquat Org 12:129–134

Cochennec N, LeRoux F, Berthe F, Gerard A (2000) Detection of *Bonamia ostreae* based on small subunit ribosomal probe. J Invertebr Pathol 76:26–32

Cochennec-Laureau N, Auffret M, Renault T, Langlade A (2003) Changes in circulating and tissue-infiltrating hemocyte parameters of European flat oysters, *Ostrea edulis,* naturally infected with *Bonamia ostreae*. J Invertebr Pathol 83:23–30

Culloty S, Mulcahy M (2007) *Bonamia ostreae* in the native oyster *Ostrea edulis:* a review. Marine Environment and Health Series No. 29. Marine Institute, Rinville

Culloty SC, Novoa B, Pernas M, Longshaw M, Mulcahy MF, Feist SW, Figueras A (1999) Susceptibility of a number of bivalve species to the protozoan parasite *Bonamia ostreae* and their ability to act as vectors for this parasite. Dis Aquat Org 37:73–80

Culloty S, Cronin M, Mulcahy M (2003) Possible limitations of diagnostic methods recommended for the detection of the protistan, *Bonamia ostreae* in the European flat oyster, *Ostrea edulis.* Bull Eur Assoc Fish Pathol 23:67–71

Da Silva PM, Villalba A (2004) Comparison of light microscopic techniques for the diagnosis of the infection of the European flat oyster *Ostrea edulis* by the protozoan *Bonamia ostreae.* J Invertebr Pathol 85:97–104

Diggles BK, Cochennec-Laureau N, Hine PM (2003) Comparison of diagnostic techniques for *Bonamia exitiosus* from flat oysters *Ostrea chilensis* in New Zealand. Aquaculture 220:145–156

Hine PM, Cochennec-Laureau N, Berthe FCJ (2001) *Bonamia exitiosus* n. sp. (Haplosporidia) infecting flat oysters *Ostrea chilensis* in New Zealand. Dis Aquat Org 47: 63–72

Howard DW, Lewis EJ, Kelleher BJ, Smith CS (eds) (2004) Histological techniques for marine bivalve molluscs and crustaceans. NOAA Tech Memo NOS NCCOS 5. NOAA, Oxford, MD

Lallias D, Arzul I (2008) *Bonamia ostreae*-induced mortalities in one-year old European flat oysters *Ostrea edulis*: experimental infection by cohabitation challenge. Aquat Living Resour 21:423–439

Lightner D, Redman R (1998) Shrimp diseases and current diagnostic methods. Aquaculture 164:201–220

Lynch S, Armitage D, Wylde S (2005) The susceptibility of young prespawning oysters, *Ostrea edulis*, to *Bonamia ostreae.* J Shellfish Res 24:1019–1025

Lynch SA, Mulcahy MF, Culloty SC (2008) Efficiency of diagnostic techniques for the parasite, *Bonamia ostreae*, in the flat oyster, *Ostrea edulis.* Aquaculture 281:17–21

Lynch SA, Abollo E, Ramilo A, Cao A, Culloty SC, Villalba A (2010) Observations raise the question if the Pacific oyster, *Crassostrea gigas*, can act as either a carrier or a reservoir for *Bonamia ostreae* or *Bonamia exitiosa*. Parasitology 137:1515–1526

Lynch SA, Villalba A, Abollo E, Engelsma M, Stokes NA, Culloty SC (2013) The occurrence of haplosporidian parasites, *Haplosporidium nelsoni* and *Haplosporidium* sp., in oysters in Ireland. J Invertebr Pathol 112:208–212

Marty GD, Bower SM, Clarke KR, Meyer G and others (2006) Histopathology and a real-time PCR assay for detection of *Bonamia ostreae* in *Ostrea edulis* cultured in western Canada. Aquaculture 261:33–42

Narcisi V, Arzul I, Cargini D, Mosca F and others (2010) Detection of *Bonamia ostreae* and *B. exitiosa* (Haplosporidia) in *Ostrea edulis* from the Adriatic Sea (Italy). Dis Aquat Org 89:79–85

OIE (World Organisation for Animal Health) (2012) Manual of diagnostic tests for aquatic animals. OIE, Paris

O'Neill G, Culloty S, Mulcahy M (1998) The effectiveness of two routine diagnostic techniques for the detection of the protozoan parasite *Bonamia ostreae* (Pichot et al. 1980). Bull Eur Assoc Fish Pathol 18:117–120

Sambrook J, Russell D (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schlötterer C (2004) The evolution of molecular markers — just a matter of fashion? Nat Rev Genet 5:63–69

Shaw B, Battle H (1957) The gross and microscopic anatomy of the digestive tract of the oyster, *Crassostrea virginica* (Gmelin). Can J Zool 35:325–347

Van Banning P (1987) Further results of the *Bonamia ostreae* challenge tests in Dutch oyster culture. Aquaculture 67: 191–194

Walker P, Subasinghe RP (2000) DNA-based molecular diagnostic techniques: research needs for standardization and validation of the detection of aquatic animal pathogens and diseases. Fish Tech Pap 395. FAO, Rome

Walsh PS, Metzger DA, Higuchi R (1991) ChelexR 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10:506–513

Zabaleta A, Barber B (1996) Prevalence, intensity, and detection of *Bonamia ostreae* in *Ostrea edulis* L. in the Damariscotta River area, Maine. J Shellfish Res 15:395–400

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