Preventive Veterinary Medicine
October 2020, Volume 183, Pages 105126 (8p.)
https://doi.org/10.1016/j.prevetmed.2020.105126
https://archimer.ifremer.fr/doc/00643/75523/

Archimer
https://archimer.ifremer.fr

A new multiplex real-time pcr assay to improve the diagnosis of shellfish regulated parasites of the genus marteilia and bonamia

Canier Lydie [1, *], Dubreuil Christine [1], Noyer Mathilde [1], Serpin Delphine [1], Chollet Bruno [1], Garcia Celine [1], Arzul Isabelle [1]

[1] Ifremer, RBE-SG2M-LGPMM, Station de La Tremblade, Avenue de Mus de Loup, F-17390 La Tremblade, France

* Corresponding author : Lydie Canier, email address : Lydie.Canier@ifremer.fr

Abstract :

Aquaculture including shellfish production is an important food resource worldwide which is particularly vulnerable to infectious diseases. Marteilia refringens, Bonamia ostreae and Bonamia exitiosa are regulated protozoan parasites infecting flat oysters Ostrea edulis that are endemic in Europe. Although some PCR assays have been already developed for their detection, a formal validation to assess the performances of those tools is often lacking. In order to facilitate the diagnosis of flat oyster regulated diseases, we have developed and evaluated a new multiplex Taqman® PCR allowing the detection of both M. refringens and Bonamia sp. parasites in one step.

First part of this work consisted in assessing analytical sensitivity and specificity of the new PCR assay. Then, diagnostic performances were assessed by testing a panel of field samples with the new real-time PCR and currently recommended conventional PCR methods for the detection of M. refringens and Bonamia sp. Samples were collected from the main flat oyster production sites in France (N = 386 for M. refringens and N = 349 for B. ostreae). In the absence of gold standard, diagnostic sensitivity and specificity of the new PCR were estimated through Bayesian latent class analysis (DSe 87,2% and DSp 98,4% for the detection M. refringens, DSe 77,5% and DSp 98,4% for the detection of Bonamia sp.). Those results suggest equivalent performances for the detection of Bonamia sp. and an improved sensitivity for the detection of M. refringens compared to commonly used conventional protocols. Finally, the new PCR was evaluated in the context of an inter-laboratory comparison study including 17 European laboratories. Results revealed a very good reproducibility with a global accordance (intra-laboratory precision) >96% and a global concordance (inter-laboratory precision) >93% for both targets, demonstrating that this new tool is easily transferable to different laboratory settings.

This is the first assay designed to detect both Marteilia refringens and Bonamia sp. in a single step and it should allow reducing the number of analysis to monitor both diseases, and where relevant to demonstrate freedom from infection.

Please note that this is an author-produced PDF of an article accepted for publication following peer review. The definitive publisher-authenticated version is available on the publisher Web site.

Highlights

► *M. refringens, B. ostreae* and *B. exitiosa* are regulated parasites of flat oysters. ► First validated PCR detecting all regulated pathogens of flat oysters in one step. ► Multiplex PCR performances similar or higher than currently recommended PCR assays. ► Represents a substantial improvement in time, resources and accuracy. ► Useful to monitors parasites prevalence or for demonstration of freedom.

Keywords : multiplex real-time PCR, Bonamia spp., Marteilia refringens, flat oysters, diagnostic accuracy study, Inter laboratory comparison

Please note that this is an author-produced PDF of an article accepted for publication following peer review. The definitive publisher-authenticated version is available on the publisher Web site.

Abbreviations

DSe: Diagnostic sensitivity

DSp: Diagnostic specificity

EURL : European Union Reference Laboratory

OIE: World Organization for Animal Health

PCR: Polymerase Chain Reaction

ILC: Inter Laboratory Comparison

Keywords : multiplex real-time PCR, *Bonamia* spp., *Marteilia refringens*, flat oysters, diagnostic accuracy study, Inter laboratory comparison

Journal Pre-proof

INTRODUCTION

Shellfish contribute to food security word wild and have an important function in the marine environment. Diseases are a major threat for these productions as well as wild populations. Marteiliosis and Bonamiosis are two diseases caused by protozoan parasites that have resulted in mass mortality events of flat oysters in Europe and in different parts of the world (Berthe et al., 2004; Cranfield et al., 2005; Hudson and Hill, 1991; Montes, 1990; Van Banning, 1991).Here after, Marteiliosis and Bonamiosis is used for infection with *M. refringens*, and with *B. ostreae* and *B. exitiosa*, respectively.

Journal Pre-proof

Marteilia refringens belongs to the order Paramixida. It was first detected in France in the 60's associated with mortalities of European flat oysters *Ostrea edulis* (see Grizel et al., 1974) and since then has been observed in several other European countries. The parasite generally infects the epithelium of the digestive system (see Grizel et al., 1974). Death of *O. edulis* usually occurs during the second year after the initial infection in association with the sporulation of the parasite. *Marteilia refringens* is also known to infect mussels *Mytilus edulis* and *Mytilus galloprovincialis* (see Tige and Rabouin, 1976; Villalba et al., 1993), although the induced disease is less severe than in flat oysters. However, whether Marteiliosis in mussels and in flat oysters is causes by the same parasite is not clear. Two distinct species were recognized: *M. refringens* infecting oysters and *M. maurini* infecting mussels (See Comps et al., 1981; Figueras and Montes, 1988; Le Roux et al. 2001). Further studies suggested that morphological and genomic differences between *M. refringens* and *M. maurini* were too low to consider them as separate species (Balseiro et al., 2007; Longshaw at al., 2001; Lopez-Flores et al., 2004), and both species were officially named *M. refringens* in 2007. Two types of *M. refringens* were then recognized: the O-type, more often detected in flat oysters, and the M-type more often detected in mussels (*M. refringens* definition according to OIE Aquatic Manual, 2008-2019). More recently, a taxonomic study using high throughput sequencing suggested restoring the two separate species recognition (Kerr et al., 2018). Authors proposed to rename the M-type as *Marteilia pararefringens* (Kerr et al., 2018). In this study, the "O-type" and "M-type" nomenclature will be used. *M. refringens* has also been sporadically reported in other bivalve species such as clams *Solen marginatus* and *Chamelea gallina* (Lopez-Flores et al., 2008a; Lopez-Flores et al., 2008b), and the mussel *Xenostrobus secures* (Pascual et al., 2010). Other *Marteilia* species have been described in marine molluscs including *M. sydneyi* in Sydney rock oysters *Saccostrea glomerata* in Australia (Perkins and Wolf, 1976), *M. cochillia* in cockles *Cerastoderma edule* in Spain (Carrasco et al., 2013; Villalba et al., 2014), *M.*

Journal Pre-proof

octospora in clams *Solen marginatus* (Ruiz et al., 2016), *M. granula* and *M. tapetis* in Manila clams *Ruditapes phillipinarum* from Japan (Itoh et al., 2014) and South Korea (Kang et al., 2019), respectively.

Bonamiosis is caused by parasites of the genus *Bonamia* which are small-size eukaryotes also called microcells belonging to the order Haplosporidia. *Bonamia* parasites mainly infect flat oysters of the genus *Ostrea*. They are intracellular parasites infecting hemocytes. Host death usually occurs concurrently to the highest intensity infection level. *Bonamia ostreae* was originally described in France (*Ostrea edulis*) in the 70’s (See Pichot et al., 1981) and had quickly spread along the European coasts. *Bonamia ostreae* is also present in North America (Friedman et al., 1989) and in New Zealand since 2015 (Lane et al., 2016). Conversely, *Bonamia exitiosa* was initially reported in New Zealand (*Ostrea chilensis*) and Australia (*Ostrea angasi*) in the 80’s (Corbeil et al., 2006b; Dinamani et al., 1987; Hine et al., 2001). It has been then detected in the USA (*Crassostrea ariakensis*) (Burreson et al., 2004) and in Europe since 2007 (Abollo et al., 2008). Co-infections with *B. ostreae* and *B. exitiosa* can occur in area of high prevalence (Ramilo et al., 2014; Lane et al., 2016). Another *Bonamia* species, *Bonamia perspora,* has been characterized in the flat oyster *Ostreola equestris* in North Carolina, USA (Carnegie et al., 2006).

Although bonamiosis and marteiliosis are endemic in Europe, some countries or zones are still free of *Bonamia ostreae, B. exitiosa* and/or *Marteilia refringens*, and surveillance effort is required to prevent diseases introduction into those areas. These protozoan parasites are listed as notifiable pathogens at the European level (EU directive 2006/88, EU implementing regulation 2018/1882) and at the International level (OIE Aquatic Animal Health Code 2019).

Journal Pre-proof

Journal Pre-proof

The flat oyster *Ostrea edulis* is the native European oyster species. Overfishing combined with diseases, notably marteiliosis and bonamiosis, resulted in an important decrease of flat oyster production as well as the decline of wild populations in Europe. This species is now classified as endangered all over Europe (OSPAR agreement 2008-6). Because of its importance for the marine ecosystem, there is a growing interest in restoring flat oyster' populations (Native Oyster Restoration Alliance, https://noraeurope.eu). Moreover, considering the mass mortality events affecting the predominantly farmed oyster species in Europe, the Pacific oyster *Crassostrea gigas* (EFSA, 2010), the flat oyster is an interesting candidate species for production diversification. Reviving flat oyster populations requires an efficient monitoring of diseases, including proper diagnostic tools. Histology and cytology are the traditional techniques used for the detection of *Marteilia* spp. and *Bonamia* spp. parasites, but do not allow species discrimination or the detection of low infections.

Several conventional PCR assays are available for the detection of the genus *Marteilia* (Le Roux et al., 1999; Lopez-Flores et al., 2004) or *M. refringens* (Le Roux et al., 2001), and real-time Taqman® PCR assays were recently developed to rapidly detect and type *M. refringens* (Carrasco et al., 2017)(EURL for mollusc diseases unpublished results https://www.eurl-mollusc.eu/SOPs). An even wider choice of PCR assays is available for the detection of parasites of the genus *Bonamia* and includes conventional and real-time PCR for the detection at the genus level (Carnegie et al., 2000; Cochennec et al., 2000; Corbeil et al., 2006a; Diggles et al., 2003; Marty et al., 2006) and real-time SYBR-Green PCRs for the specific detection of *B. ostreae* alone (Robert et al., 2009) and for the detection and discrimination of *B. ostreae* and *B. exitiosa* (Ramilo et al., 2013).

Although a broad range of PCR assays have been already developed, these tools have not been formally validated according to the OIE recommendations, or partly (Buss et al., 2019; Ramillo et

Journal Pre-proof

al., 2013). In order to facilitate the diagnosis of marteiliosis and bonamiosis, we have developed and validated a new multiplex Taqman® PCR assay allowing the detection of regulated pathogens of flats oysters in one step. This new assay was designed to target the 18S rDNA gene of *M. refringens* and *Bonamia sp*. It is intended to be used to monitor parasite prevalence and where relevant to demonstrate freedom from infection in flat oyster populations.

Performance of the new PCR assay were assessed following the 3-stages validation pathway recommended by the OIE (Manual of Diagnostic Tests for Aquatic animals, 2019), which consists in the assessment of 1 -analytical characteristics, 2-diagnostic characteristics, and 3-reproducibility through Inter Laboratory Comparison test. This manuscript attempts to report all key elements of the PCR evaluation according to the STRADAS-aquatic guidelines (Gardner et al., 2016).

MATERIALS AND METHODS

Field samples

Flat oyster samples used to assess diagnostic performances of the new PCR assay were collected outside of mortality events between 2011 and 2014 in three main production sites in Brittany, France (Bay of Brest, Bay of Cancale and Golfe du Morbihan). Flat oysters were screened using conventional PCR assays (protocol described in the “conventional PCRs” section below) in the context of a surveillance programme aiming at monitoring the prevalence of *M. refringens* and *B. ostreae* in France.

As *Bonamia exitiosa* is absent from the 3 previous sampling sites, the new PCR was tested on an additional set of 32 samples collected in the Mediterranean sea off the city of Sète (south of France), where *B. exitiosa* has been reported associated with mortalities.

Journal Pre-proof

Collected oysters were stored frozen before DNA extraction. Samples used to assess diagnostic performances are detailed in Table S1.

Plasmids

pUC57 plasmids containing the PCR targeted sequences of *Marteilia refringens* (5'ACGATCAAAGTGAGCTCGTGCTCGATGGTTTTGCATGGAATCGTGGAACGGGGCCGGCGTTCTGTACTTCGAGAGCGCTGGCGACGATCAACGGGAGCGATCAGGGGTGAGGGAACTG- 3') and *Bonamia* sp. (5'-CAGGATGCCCTTAGATGCTCTGGGCTGCACGCGCGCTACAATGGTGCGTTCAACGAGTTTGACCCGGCTTGACAAGGCCGGGTAATCTTCAACGCGCACCCAAGTTGGGATAGATGATTGCAATTGTTCATCTTGAACAAGGAATATCTAGTAAACGCAAGTCATCAACTTGCATTGATTACGTCCCTGCCCTTTGTAC-3') were ordered from the "gene synthesis" service of Eurogentec France.

DNA extraction

Between 20 and 25 mg of gills and digestive gland tissues were collected from each oyster. DNA was extracted using the QiaAmp DNA minikit (Qiagen) following manufacturer's instructions for tissue protocol. DNA extracts were stored frozen at -20°C between 1 to 6 years before analysis and were diluted to a final concentration of 100 ng/ µl for conventional PCR applications, or 5 ng/µl for real-time PCR applications.

Conventional PCR assays

Conventional PCR amplification of *Marteilia refringens* was performed using the ITS1 primers (Pr4 & Pr5) described by Le Roux et al. 2001. PCR reactions contained 1 µl of DNA sample (at 100 ng/µl), 1 x PCR buffer, 2.5 mM $MgCl_2$, 0.5 mM of each dNTPs, 1 µM of each primers and 2.5 unit of Taq polymerase (Goldstar, Eurogentec) in a total volume of 50 µl. PCR amplifications were carried

Journal Pre-proof

out in a thermocycler (PTC-100 MJ Research) as follows: initial denaturation at 95°C for 10 minutes followed by 30 cycles (1 min et 94 °C, 1 min at 55°C, 1 min at 72°C) and a final elongation at 72°C for 10 minutes.

Conventional PCR amplification of *Bonamia* sp. was performed using the 18S primers (BO and BOAS primers) described by Cochennec et al. (2000). PCR reactions contained 1 µl of DNA sample (at 100 ng/µl), 1 x PCR buffer, 1.25 mM MgCl2, 0.5 mM of each dNTPs, 1 µM of each primers and 2.5 unit of Taq polymerase (Goldstar, Eurogentec) in a total volume of 50 µl. PCR amplifications were carried out in a thermocycler (PTC-100 MJ Research) as follows: initial denaturation at 95°C for 5 minutes followed by 30 cycles (1 min et 94 °C, 1 min at 55°C, 1 min at 72°C) and a final elongation at 72°C for 10 minutes. Samples were considered positive when a band was observed on agarose gel at 412 bp for *M. refringens* and 304 bp for *Bonamia* sp.

New multiplex real-time PCR assay

Two pairs of primers and probes were designed on the 18S rDNA to specifically amplify *Marteilia refringens* (Mar-18S primers and probe) and *Bonamia* sp. including *Bonamia ostreae* & *Bonamia exitiosa* (Bosp2-18S primers and probe) (Table 1).

PCR reactions were performed in a 25 µl final volume containing 5µl of DNA samples (at 5 ng/µl), 1X PCR Master Mix (Brillant III Ultra-Fast, Agilent Technologies® ref: # 600881), 0.4 µM Mar-18-F primer, 0.4 µM Mar-18S-R primer, 0.3 µM Mar-18S-IN probe, 0.3 µM Bosp2-18S-F primer, 0.5 µM Bosp2-18S-R primer, 0.3 µM Bosp2-18S-IN probe. PCR amplifications were carried out in the Mx3000 thermocycler (Stratagene®) as follows: initial denaturation at 95°C for 3 minutes followed by 40 cycles at 95 °C for 15 secondes and 60°C for 1 minute. Samples were tested in duplicates and considered as positive when an expected amplification curve was observed in both replicates with Ct values below the Ct cut-off. Based on the Ct values obtained at the limit of detection (Caraguel

Journal Pre-proof

et al., 2011), Ct cut-off values were set-up at 37.5 for *M. refringens* and 37 for *Bonamia* sp.. Samples with one Ct above and one Ct below the Ct cut-off value were repeated. In the few cases where repetition could not resolve sample status, samples were considered as negative.

General quality assurance statements

All laboratory analyses were performed under quality management (ISO/IEC 17025) by experienced technicians that were trained to internal procedures. Quality and concentration of extracted DNA was assessed using a NanoDrop® spectrophotometer (Thermo Fisher Scientific). Several quality controls were systematically used to validate the DNA extraction and the PCR steps. An extraction negative control (no tissue) was included in each extraction run. A PCR negative controls (no DNA) and two PCR positive controls (one for each target) were included in each PCR run. PCR positive controls consisted in DNA from the laboratory collections, extracted from *O. edulis* known to be infected with *M. refringens* and *B. ostreae* or *B. exitiosa*. All quality controls were tested by PCR in duplicates.

***In silico* specificity**

To assess the specificity of the PCR assays *in silico*, primers and probe sequences were aligned against the following 18S rDNA sequences from the NCBI nucleotide collection: *Marteilia refringens* type O (AJ250699, AB889893) or isolated from *O. edulis* (MH304628, MH304629, MH342044), *Marteilia refringens* type M, also named *pararefringens* (AB889894, MH304630, MH304631, MH304632), *Marteilia cochillia* (AB889895, MH304633, KF278722), *Marteilia sydneyi* (AB823742), *Marteilia granula* (AB856587), *Marteilioides chungmuensis* (AB110795), *Bonamia ostreae* (AF192759, AF262995, DQ312295), *Bonamia exitiosa* (DQ312295, EU016528), *Bonamia sp*.

Journal Pre-proof

(AY542903), *Bonamia perspora* (DQ356000), *Haplosporidium nelsoni* (X74131), *Haplosporidium costale* (AF387122), *Minchinia mercenariae-like* (KY522823), *Minchinia tapetis* (AY449710).

Analytical characteristics

Analytical specificity of the new real-time PCR assay was determined by testing several samples infected with targeted parasites and samples infected with closely related parasites (Table 2). Each sample was tested in triplicates.

Analytical sensitivity (or limit of detection) was established by testing 10-fold serial dilution of plasmidic DNA ranging from 0.1 to 100,000 copies / µl. Three PCR runs containing 8 replicates of each dilution were performed resulting in 24 PCR tests for each dilution. The limit of detection was determined as the last dilution where at least 95% of the samples tested positive.

To evaluate the potential competition between both targets in case of co-infection, tests were performed on cross-serial dilutions of plasmids ranging from 10-10^5 copies/µl with the highest concentrations of *M. refringens* being mixed with the lowest concentration of *Bonamia* sp. and inversely (tested combinations *M. refringens/ Bonamia sp.:* 10^5 /10 ; 10^4 /10^2; 10^3 /10^3 ; 10^2 /10^4; 10 /10^5 copies /µl); 10 copies /µl corresponding to the limit of detection of the PCR, and 10^5 copies / µl to the “strongest” positive usually find naturally.

Repeatability and preliminary reproducibility were estimated based on results from the analytical sensitivity experiments, by evaluating within and between assay variations in Ct values of replicates, as described in ISO 5725-2.

Diagnostic characteristics (DSe and DSp)

Diagnostic performances were assessed by comparing currently recommended conventional PCR assays and the new real-time PCR one on a panel of field samples. Three groups of oysters

Journal Pre-proof

representing different levels of prevalence for each targeted parasite were selected based on historical data (sampling site, age class) and previous conventional PCR results. For *Marteilia refringens,* 386 samples were selected and included a group of 51 oysters with "high" prevalence (> 50%), a group of 235 oysters with "low" prevalence (5-10%) and a group of 100 oysters from a population known to be free of the parasite.

For *Bonamia ostreae*, 349 samples were analysed and included a group of 45 oysters with "high" prevalence (> 50%), a group of 101 oysters with "low" prevalence (5-10%), and a group of 203 with "very low" prevalence (<5%), as there is no population of flat oysters known to be free of the parasite in France.

These groups of oysters were analysed retrospectively with the new real-time PCR assay and results were compared with results from conventional PCR assays.

Statistical analysis

As conventional PCR protocols cannot be considered as "gold standards", the diagnostic sensitivity (DSe) and specificity (DSp) of the PCR assays were assessed through latent class analysis. A Bayesian approach was used, where parameters were estimated by Markov Chain Monte Carlo (MCMC) methods via Gibbs sampling. Analysis was performed as described by Joseph et al. (1995) by adapting the model to 2 methods and 3 populations. To take into account the possible dependence between PCR assays, conditional dependence was modelled using the covariance between methods (Vacek et al., 1985; Dendukuri et al. 2001) within the diseased class. Analysis was performed with non-informative priors (beta distributions (1,1)) except for the DSe and DSp of the conventional *Bonamia* sp. PCR for which estimates were available in the literature (Balseiro et al., 2006). Based on published results (Balseiro et al., 2006), beta(8.3, 1.8) priors were used for these two parameters, reflecting 95% confidence that the DSe and DSp is above 60% with a mode

Journal Pre-proof

Journal Pre-proof

set at 90%. The model was implemented in JAGS within the R statistical software environment using packages rjags. For each targeted pathogen, four Monte–Carlo Markov chains (MCMCs) were run with 15,000 iterations with the first 5,000 iterations discarded as a 'burn-in' and the subsequent 10,000 iterations retained for posterior inference. Se and Sp estimates and their 95% credibility interval are reported. The model is available in supplementary file S3.

Estimation of reproducibility: Inter Laboratory Comparison study

The European Union Reference Laboratory for mollusc diseases organized an Inter Laboratory Comparison test (ILC) in 2017, in order to test the competency of National Reference Laboratories regarding the detection of *Bonamia* sp. and *Marteilia refringens* in flat oyster (*Ostrea edulis*) by PCR. On this occasion, participants were also invited to test the new real-time PCR assay on the ILC set of samples. Primers and probes of the new PCR assay were sent to the participants, along with a set of 24 samples of flat oyster tissues. Samples were prepared by aliquoting suspensions of small pieces of digestive glands and gills from flat oysters fixed in ethanol and included 8 negative samples, 8 *Marteilia refringens* positive samples, and 8 *Bonamia ostreae* positive samples (Fig. S1). Expected answers were qualitative (detected / not detected). Reference results were established based on historical data and results obtained with different methods including several real-time PCRs and histology for some samples. Percentages of matching results were calculated by comparing reference results and participant's results.

The reproducibility of the new real-time PCR assay was assessed based on the results of 17 participating laboratories by measuring the parameters described by Langton et al. 2002 (Langton et al., 2002): Accordance, Concordance and Odds ratio. Accordance is the percentage of chance to obtain same results for two identical samples tested in the same laboratory; it is a measure of "within laboratory variation". Concordance is the percentage of chance to obtain similar results for

Journal Pre-proof

two identical samples tested by two different laboratories; it is a measure of "between laboratory variation".

RESULTS

In silico specificity of primers and probes

Mar-18S primers and probe showed 100% similarity with all the 18S rDNA tested sequences from *Marteilia refringens* type O and type M, but also with some *M. cochillia* sequences. Another *M. cochillia* sequence showed one mismatch (on the Mar-18S-F primer). Two mismatches were noted with *Marteilia sydneyi* (one on the Mar-18S-F primer and one on the Mar-18S-R primer). *Marteilia granula* or *Marteilioides chungmuensis* sequences displayed about 15 mismatches distributed along the probe, the forward and the reverse primers.

Bosp2-18S primers and probe showed 100% similarity with all the 18S rDNA tested sequences from *Bonamia ostreae, Bonamia exitiosa* and *Bonamia perspora*. Sequence divergences above 25 % were observed between the probe sequence and *Haplosporidium nelsoni*, *Haplosporidium costale*, *Minchinia* sp., and *Minchinia tapetis* 18S rDNA sequences as well as at least 5 mismatches on the primer sequences.

Analytical specificity

No PCR detection was observed in uninfected flat oysters (*O. edulis*) and mussels (*Mytius edulis* and *M. galloprovincialis*). *Marteilia refringens* primers and probe were able to detect *M. refringens* type M and type O in flat oysters (*O. edulis*) and in mussels (*M. edulis and M. galloprovincialis*), but also *M. cochillia* in cockles (*Cerastoderma edule*) and *M. sydneyi* in Sydney rock oysters (*Saccostrea glomerata*). Those results are consistent with the *in silico* analysis which showed low sequence

Journal Pre-proof

Journal Pre-proof

divergences between Mar-18S primers & probe and *M. cochillia* and *M. sydneyi* 18S rDNA. No amplification was observed on samples infected with *Marteilioides chungmuensis* (Table 2). *Bonamia* sp. primers were only able to detect *B. ostreae* and *B. exitiosa*. Other tested parasites (*Haplosporidium nelsoni, Mikrocytos mackini, Mikrocytos sp.)* resulted in negative PCR results (Table 2).

Analytical sensitivity (limit of detection).

The new real-time PCR assay consistently amplified plasmidic DNA over a 5 log range from 10^5 to 10 copies /µl (table 3).The lowest dilution producing consistent positive results was 10 copies of plasmids (or genome equivalent) per µl for each targeted pathogen.

All tested combinations of mixed plasmids (*Bonamia* sp. and *M. refringens)* were correctly detected by the new PCR (data not shown).

Cut-off determination

The mean Ct value obtained at the limit of detection (10 copies / µl) was 37.8 for *M. refringens* and 36.9 for *Bonamia* sp. Therefore, Ct cut-off was established at 37.5 for *M. refringens* and 37 for *Bonamia* sp.

Repeatability and preliminary reproducibility (or within-laboratory precision)

Repeatability and preliminary reproducibility were assessed based on Ct values from the analytical sensitivity experiments (Table 3). Higher intra-assay variability was observed for *Marteilia refringens* than for *Bonamia* sp. amplifications. Intermediate precision varied according to the tested dilution and tended to be higher for smaller DNA concentrations. Variation coefficients of

Journal Pre-proof

the intermediate precision were always below 5%, for each tested dilution (Table 3), suggesting a good intra-laboratory precision of the PCR assay.

Diagnostic performances (Diagnostic sensitivity and specificity)

A representative panel of field samples was analysed with the new real-time PCR assay and currently recommended conventional PCR ones (Le Roux et al., 2001, Cochennec et al., 2000) (Cochennec et al., 2000; Le Roux et al., 2001). Results are presented in Table 4A.

Concerning *M. refringens* detection, both the conventional (Le Roux et al., 2001) and the new real-time PCR did not yield positive results in oyster samples from the population known to be free of the parasite. The new real-time PCR detected more *M. refringens* positive samples in the "high" and "low" prevalence groups than the conventional PCR (table 4A).

Concerning *Bonamia* sp. detection, the new real-time PCR and the conventional PCR (Cochennec et al., 2000) yielded similar results; the conventional protocol detected 3 additional *B. ostreae* infections in the "high" prevalence group, and the new real-time protocol detected 2 additional *B. ostreae* infections in the "low" prevalence group (table 4A).

Diagnostic sensitivity (DSe) and specificity (DSp) were estimated using Bayesian latent class analysis (table 4B). For the detection of *M. refringens*, the new real-time PCR has an estimated DSe of 87.2% which is higher than for the conventional PCR (60.7%), and an estimated Dsp of 98.4% which is equivalent to the conventional PCR. For the detection of *B. ostreae*, the new real-time PCR has an estimated DSe of 77.5% which is slightly lower than for the conventional PCR (82.8%), and an estimated Dsp of 98.4% which is equivalent to the conventional PCR.

Journal Pre-proof

As *Bonamia exitiosa* is absent from the three previous sampling sites, an additional set of samples coming from a site where *B. exitiosa* has been detected was tested with the conventional PCR and the new real-time PCR (Table 5).

Results showed 90.6 % of agreement between both methods. Three samples yielded discordant results: two were samples found positive with the new real-time PCR and negative with the conventional PCR, and, conversely, one sample was found positive with the conventional PCR and negative with the new PCR.

Inter Laboratory Comparison Study

Seventeen laboratories tested a set of 24 samples of flat oyster tissues with the new real-time PCR assay and with their own routine PCR assays (sample preparation detailed in Fig. S1). Results obtained with the new real-time PCR during this Inter Laboratory Comparison study are summarized in Table 6. Participants obtained an average of 98.3% of matching results for the detection of *Marteilia refringens* and 96.1% for the detection of *Bonamia* sp. with the new real-time PCR. Almost all positive samples (99.3%) were correctly detected by the new real-time PCR assay. Errors mainly corresponded to negative samples identified as positive, especially for *B. ostreae* (the rate of false positives was 2.2% for *M. refringens* and 5.5% for *Bonamia* sp.).

Method precision of the new real-time PCR was estimated for infected and non-infected samples (Table 7). Global accordance and concordance were very high for both the detection of *Marteilia refringens* (>95%) and *Bonamia* sp. (> 93%) demonstrating a good repeatability and reproducibility of the new assay. Odds ratios calculated on these data showed a higher degree of “between laboratory variation” for non-infected samples, indicating a lower reproducibility for negative

Journal Pre-proof

samples. In particular, the concordance for the detection of *Bonamia* sp. was below 90% on non-infected samples which is explained by the occurrence of false positives in some laboratories.

DISCUSSION

Microscopic and molecular methods are complementary tools for the surveillance of mollusc diseases. Microscopy allows the detection of a large range of pathogens, in particular protozoan parasites, as well as the observation of tissues lesions, giving information on the general health status of mollusc populations. PCR, and especially real-time PCR, are sensitive tools allowing the rapid detection of a specific pathogen; they are particularly suited for the targeted surveillance of listed pathogens such as *Bonamia ostreae*, *B. exitiosa* and *Marteilia refringens*. Currently, molecular diagnosis of regulated pathogens of flat oysters is still mainly performed using conventional PCR protocols developed in the 2000's (survey performed by the EURL for mollusc diseases in 2017) that were not formally validated. Laboratories are progressively shifting from conventional to real-time PCR which provide several benefits such as a reduced time of analysis, an increased sensitivity and a decreased cross-contamination risk. Multiplex PCR allowing the detection of several pathogens in one reaction are quite uncommon for the diagnosis of mollusc diseases. In this context, we have developed a new real-time PCR assay allowing the detection of both *Marteilia* sp. and *Bonamia* sp. parasites in flat oysters in an easy single step. Performances of the new real-time PCR assay were assessed following the validation pathway recommended by the OIE (Manual of Diagnostic Tests for Aquatic animals, 2019).

The new real-time PCR assay was shown to detect both *Bonamia ostreae* and *B. exitiosa* and not *Haplosporidium nelsoni*, *Mikrocytos mackini*, and *Mikrocytos donaxi*. *In silico* analyses suggest that it would probably detect *B. perspora* but not other closely related mollusc parasites belonging to

Journal Pre-proof

the genera *Haplosporidium*, *Minchinia* and *Mikrocytos*. The new PCR assay was demonstrated to detect both *Marteilia refringens* type M and type O in flat oysters *O. edulis* and in mussels *Mytilus edulis* and Mytilus *galloprovinciallis*. In tested samples, it also detected *M. cochillia*, a parasite associated with high mortalities of cockles in Spain since 2012, (Carrasco et al., 2013; Villalba et al., 2014) and *M. sydneyi*, a parasite causing recurring mass mortalities of Sydney rock oysters in Australia (Perkins and Wolf, 1976). The close proximity of 18S rDNA sequences of those parasite species with *M. refringens* (Villalba et al., 2014) explains these results. *Martelioides chungmuensis* was not detected by the new assay and *Marteilia granula* should not be detected considering the sequence divergence observed on primers and probes sequences. *Ostrea edulis* is not considered as susceptible to *M. cochillia* and *M. sydneyi*, therefore, detection of *Marteilia* sp. with the new real-time PCR strongly indicate the presence of *M. refringens*. In case of first detection with the new real-time PCR in a new place, further characterisation would be required to specify the parasite species.

The analysis of serial dilutions of plasmidic suspensions showed that the new assay can detect down to 10 copies of each targeted DNA per µl. Information on the limit of detection (in copies / µl) of other *M. refringens* or *Bonamia* PCR methods is not available , except for the SYBR Green PCR for the detection of *B. ostreae* (Robert et al., 2009) whose detection limit is also 10 copies / µl. Additional experiments with different concentrations of mixed plasmids (*Bonamia* sp. and *M. refringens*), suggested that the presence of one target would not impact the detection of the other target in case of co-infection. To compare the analytical sensitivity of the new real-time PCR with other existing real-time PCRs, we tested serial dilutions of flat oyster DNA infected with *M. refringens* type M, *M. refringens* type O, *B. ostreae* and *B. exitiosa* with the new real-time PCR, the SYBR Green PCR *B. ostreae /B. exitiosa* (Ramilo et al., 2013) and the Taqman® PCR *M. refringens*

Journal Pre-proof

Type M / type O (EURL unpublished results, https://www.eurl-mollusc.eu/SOPs) (data not shown). All real-time PCR assays consistently detected parasites down to 4 log dilutions, and showed no amplification over 5 log dilutions, suggesting similar detection limits.

The new real-time PCR was compared to OIE-recommended conventional PCRs (Cochennec et al., 2000; Le Roux et al., 2001) on a panel of field samples categorised in 3 prevalence groups. Tested oysters were collected outside mortality events with a focus on "low" or "very low" prevalence groups to represent the spectrum of infection when monitoring apparently healthy populations. Compared to the conventional *M. refringens* PCRs (Le Roux et al., 2001), the new real-time PCR has a higher sensitivity (DSe new PCR: 87.2%, DSe conventional PCR: 60.7%) and an equivalent specificity (98-99%) and should allow a better detection of low *Marteilia* sp. infections. Compared to the conventional *Bonamia sp.* PCR (Cochennec et al., 2000), the new real-time PCR has an equivalent sensitivity (77.5-82.8%) and specificity ((98-99%). Existing molecular tools for the diagnosis of *Marteilia* parasites were rarely assessed for their diagnostic sensitivity and specificity, however, some data are available for tools targeting *Bonamia* parasites. The conventional *Bonamia* sp. PCR (Cochennec et al., 2000) was compared to histology and cytology on a set of 240 oysters, and showed en estimated sensitivity of 92% and specificity between 85-90% (Balseiro et al, 2006). The SYBR Green PCR for the detection of *B. ostreae* and *B. exitiosa* (Ramilo et al., 2013) was compared to histology and conventional PCR-RFLP (Cochennec et al., 2000) on a set of flat oyster (N=137). Estimated sensitivity of the SYBR Green PCR was very high (99% for *B. ostreae* and 100% for *B. exitiosa*), however, estimated specificity was quite low (72% for *B. ostreae* and 77% for *B. exitiosa*). More recently, real-time PCR (Corbeil et al., 2006), histology and cytology were evaluated for their detection of *Bonamia* sp. in Australian farmed oysters (N=400)(Buss et al. 2019). Sensitivity and specificity of the real-time PCR was estimated at 69% and 93% respectively.

Journal Pre-proof

The new PCR was successfully implemented by 17 laboratories that tested a set of 24 samples of known status in the context of an Inter Laboratory Comparison (ILC) study. Almost all positive samples (99.3%) were correctly detected by the participants, suggesting a high relative sensitivity of the new assay. Most of incorrect results consisted in false positives for both pathogens; and more particularly *B. ostreae,* even though the rate of false positives was low (5.5% for *B. ostreae,* 2.2% for *M. refringens)*. This observation is in agreement with the 98.4% DSp obtained for *M. refringens* with the Bayesian model, but suggests that the 98.4% DSp for *B. ostreae* may be lower when the PCR assay is used by several laboratories. Precision estimates, including accordance (within laboratory precision) and concordance (between laboratories precision), were always above 90% for both targeted pathogens, demonstrating a very good robustness of the new real-time PCR assay.

In this study, a Ct cut-off approach was used to determine sample status; positive or negative. A Ct above the cut-off value could be a false positive due to contamination or unspecific amplification. However a Ct above the cut-off value might also be obtained in case of very low infection or reflect the presence of parasites DNA in the environment. Indeed, oysters are filter feeders that could accumulate those environmental DNAs. Therefore test interpretation should differ for diagnosis in endemic areas where the cut off approach is recommended and disease certification for translocation to free zones where late PCR amplification would require more investigation.

CONCLUSION

We have developed and validated for the first time a real-time PCR assay allowing to simultaneously detect *Bonamia* sp. and *Marteilia* sp. in flat oysters *Ostrea edulis.* This new tool

Journal Pre-proof

should allow reducing the number of analysis to perform to monitor regulated pathogens of flat oysters (*M. refringens*, *B. ostreae* and *B. exitiosa*) and represents a much quicker and more cost-effective alternative to existing PCRs, with equivalent or better performances. Moreover, inter-laboratory comparison showed that the new PCR is robust and easily transferable to other laboratory settings.

It would be particularly useful to monitor the prevalence of both Marteilia and Bonamia parasites in endemic areas and to demonstrate freedom from regulated pathogens in flat oyster populations.

Journal Pre-proof

Journal Pre-proof

AKNOWLEDGEMENTS

This work was financially supported by Ifremer, by EU DG SANTE through the European Reference Laboratory for mollusc diseases, and by DGAL through the National Reference Laboratory for Mollusc Diseases.

Authors thank L. Reddecliff for providing *Saccostrea commercialis* infected with *Marteilia sydneyi*, N. Carrasco for providing *Cerastoderma edule* infected with *Marteilia cochillia,* N. Itoh for providing *Crassostrea gigas* infected with *Marteiloïdes chungmuensis* and G. Meyer for providing *Ostrea edulis* infected with *Mikrocytos mackini*.

S. Audic is aslo acknowledged for his assistance on Bayesian latent class analysis.

Authors acknowledge all the laboratories that participated in the Inter Laboratory comparison Study:

Snjezana Zrncic, Croatian Veterinary Institute, Croatia

Lone Madsen, DTU National Veterinary Institute, Denmark

Michael Gubbins, CEFAS, England, UK

Giuseppe Arcangeli,Istituto Zooprofilattico Sperimentale delle Venezie, Italy

Deborah Cheslett and Teresa Morrissey, Marine Institute, Ireland

Céline Garcia and Delphine Serpin, IFREMER, LGPMM, France

Hannah Stagg, Marine Scotland, Science Marine Laboratory, Scotland, UK

Sven Bergmann, Friedrich-Loeffler-Institut, Germany

Dimitris Karagiannis, Directorate of Veterinary Centre of Thessaloniki, Greece

Bojan Adzic, Diagnostic Veterinary Laboratory, Montenegro

Paul Savage, Agri-Food and Biosciences Institute, Northern Ireland

Mihaela Costea, Institute for Diagnosis and Animal Health, Romania

Darja Kusar, Veterinary Faculty, Institute of Microbiology and Parasitology, Slovenia

Journal Pre-proof

Journal Pre-proof

Anders Alfjorden and Mats Isaksson, National Veterinary Institute, Sweden

Olga Haenen and Michal Voorbergen,WBVR of Wageningen UR, The Netherlands

Oznur Yazicioglu, Bornova Veterinary Control Institute, Turkey

Journal Pre-proof

Journal Pre-proof

References

Abollo, E., Ramilo, A., Casas, S.M., Comesaña, P., Cao, A., Carballal, M.J., Villalba, A., 2008. First detection of the protozoan parasite Bonamia exitiosa (Haplosporidia) infecting flat oyster Ostrea edulis grown in European waters. Aquaculture 274, 201-207.

Balseiro, P., Montes, A., Ceschia, G., Gestal, C., Novoa, B., Figueras Huerta, A., 2007. Molecular epizootiology of the European Marteilia spp., infecting mussels (Mytilus galloprovincialis and M. edulis) and oysters (Ostrea edulis): an update.

Berthe, F.C., Le Roux, F., Adlard, R.D., Figueras, A., 2004. Marteiliosis in molluscs: a review. Aquatic Living Resources 17, 433-448.

Burreson, E.M., Stokes, N.A., Carnegie, R.B., Bishop, M.J., 2004. Bonamia sp.(Haplosporidia) found in nonnative oysters Crassostrea ariakensis in Bogue Sound, North Carolina. Journal of Aquatic Animal Health 16, 1-9.

Caraguel, C.G., Stryhn, H., Gagné, N., Dohoo, I.R., Hammell, K.L., 2011. Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose: analytical and epidemiologic approaches. Journal of Veterinary Diagnostic Investigation 23, 2-15.

Carnegie, R.B., Barber, B.J., Culloty, S.C., Figueras, A.J., Distel, D.L., 2000. Development of a PCR assay for detection of the oyster pathogen Bonamia ostreae and support for its inclusion in the Haplosporidia. Diseases of Aquatic Organisms 42, 199-206.

Carnegie, R.B., Burreson, E.M., Mike Hine, P., Stokes, N.A., Audemard, C., Bishop, M.J., Peterson, C.H., 2006. Bonamia perspora n. sp.(Haplosporidia), a parasite of the oyster Ostreola equestris, is the first Bonamia species known to produce spores. Journal of Eukaryotic Microbiology 53, 232-245.

Carrasco, N., Hine, P.M., Durfort, M., Andree, K.B., Malchus, N., Lacuesta, B., Gonzalez, M., Roque, A., Rodgers, C., Furones, M.D., 2013. Marteilia cochillia sp nov., a new Marteilia species affecting the edible cockle Cerastoderma edule in European waters. Aquaculture 412, 223-230.

Journal Pre-proof

Journal Pre-proof

Carrasco, N., Voorbergen-Laarman, M., Lacuesta, B., Furones, D., Engelsma, M.Y., 2017. Application of a competitive real time PCR for detection of Marteilia refringens genotype “O” and “M” in two geographical locations: The Ebro Delta, Spain and the Rhine-Meuse Delta, the Netherlands. Journal of invertebrate pathology 149, 51-55.

Cochennec, N., Le Roux, F., Berthe, F., Gerard, A., 2000. Detection of Bonamia ostreae based on small subunit ribosomal probe. Journal of Invertebrate Pathology 76, 26-32.

Corbeil, S., Arzul, I., Diggles, B., Heasman, M., Chollet, B., Berthe, F.C.J., Crane, M.S., 2006a. Development of a TaqMan PCR assay for the detection of Bonamia species. Diseases of Aquatic Organisms 71, 75-80.

Corbeil, S., Arzul, I., Robert, M., Berthe, F.C., Besnard-Cochennec, N., Crane, M.S.J., 2006b. Molecular characterisation of an Australian isolate of Bonamia exitiosa. Diseases of Aquatic Organisms 71, 81-85.

Cranfield, H., Dunn, A., Doonan, I., Michael, K., 2005. Bonamia exitiosa epizootic in Ostrea chilensis from Foveaux Strait, southern New Zealand between 1986 and 1992. ICES Journal of Marine Science 62, 3-13.

Diggles, B., Cochennec-Laureau, N., Hine, P., 2003. Comparison of diagnostic techniques for Bonamia exitiosus from flat oysters Ostrea chilensis in New Zealand. Aquaculture 220, 145-156.

Dinamani, P., Hine, P.M., Jones, J.B., 1987. Occurrence and Characteristics of the Hemocyte Parasite Bonamia Sp in the New-Zealand Dredge Oyster Tiostrea-Lutaria. Diseases of Aquatic Organisms 3, 37-44.

EFSA Panel on Animal Health and Welfare (AHAW), 2010. Scientific Opinion on the increased mortality events in Pacific oysters, Crassostrea gigas. EFSA Journal 8, 1894.

Friedman, C., McDowell, T., Groff, J., Hollibaugh, J., Manzer, D., Hedrick, R., 1989. Presence of Bonamia ostreae among populations of the European flat oyster, Ostrea edulis Linne. California, USA. J Shellfish Res 8, 133-137.

Gardner, I.A., Whittington, R.J., Caraguel, C.G., Hick, P., Moody, N.J., Corbeil, S., Garver, K.A., Warg, J.V., Arzul, I., Purcell, M.K., 2016. Recommended reporting standards for test accuracy studies of infectious diseases of finfish, amphibians, molluscs and crustaceans: the STRADAS-aquatic checklist. Diseases of aquatic organisms 118, 91-111.

Journal Pre-proof

Grizel, H., Comps, M., Bonami, J.R., F, Cousserans, F, Duthoit J.L., Le Pennec M.A., L., 1974. Recherche sur l'agent de la maladie de la glande digestive de Ostrea edulis Linné. Science et Pêche (0036-8350) (ISTPM), 1974 , N. 240 , P. 7-30 240.

Hine, P.M., Cochennec-Laureau, N., Berthe, F.C.J., 2001. Bonamia exitiosus n. sp (Haplosporidia) infecting flat oysters Ostrea chilensis in New Zealand. Diseases of Aquatic Organisms 47, 63-72.

Hudson, E., Hill, B., 1991. Impact and spread of bonamiasis in the UK. Aquaculture 93, 279-285.

Itoh, N., Yamamoto, T., Kang, H.S., Choi, K.S., Green, T.J., Carrasco, N., Awaji, M., Chow, S., 2014. A Novel Paramyxean Parasite, Marteilia granula sp nov (Cercozoa), from the Digestive Gland of Manila Clam Ruditapes philippinarum in Japan. Fish Pathology 49, 181-193.

Joseph, L., Gyorkos, T.W., Coupal, L., 1995. Bayesian estimation of disease prevalence and the parameters of diagnostic tests in the absence of a gold standard. Am J Epidemiol 141, 263-272.

Kang, H.S., Itoh, N., Limpanont, Y., Lee, H.M., Whang, I., Choi, K.S., 2019. A novel paramyxean parasite, Marteilia tapetis sp. nov. (Cercozoa) infecting the digestive gland of Manila clam Ruditapes philippinarum from the southeast coast of Korea. J Invertebr Pathol 163, 86-93.

Kerr, R., Ward, G.M., Stentiford, G.D., Alfjorden, A., Mortensen, S., Bignell, J.P., Feist, S.W., Villalba, A., Carballal, M.J., Cao, A., Arzul, I., Ryder, D., Bass, D., 2018. Marteilia refringens and Marteilia pararefringens sp nov are distinct parasites of bivalves and have different European distributions. Parasitology 145, 1483-1492.

Lane, H.S., Webb, S.C., Duncan, J., 2016. Bonamia ostreae in the New Zealand oyster Ostrea chilensis: a new host and geographic record for this haplosporidian parasite. Diseases of Aquatic Organisms 118, 55-63.

Langton, S., Chevennement, R., Nagelkerke, N., Lombard, B., 2002. Analysing collaborative trials for qualitative microbiological methods: accordance and concordance. International Journal of Food Microbiology 79, 175-181.

Le Roux, F., Audemard, C., Barnaud, A., Berthe, F., 1999. DNA probes as potential tools for the detection of Marteilia refringens. Marine Biotechnology 1, 588-597.

Journal Pre-proof

Journal Pre-proof

Le Roux, F., Lorenzo, G., Peyret, P., Audemard, C., Figueras, A., Vivares, C., Gouy, M., Berthe, F., 2001. Molecular evidence for the existence of two species of Marteilia in Europe. J Eukaryot Microbiol 48, 449-454.

Lopez-Flores, I., De La Herran, R., Garrido-Ramos, M.A., Navas, J.I., Ruiz-Rejon, C., Ruiz-Rejon, M., 2004. The molecular diagnosis of Marteilia refringens and differentiation between Marteilia strains infecting oysters and mussels based on the rDNA IGS sequence. Parasitology 129, 411-419.

Lopez-Flores, I., Garrido-Ramos, M.A., de la Herran, R., Ruiz-Rejon, C., Ruiz-Rejon, M., Navas, J.I., 2008a. Identification of Marteilia refringens infecting the razor clam Solen marginatus by PCR and in situ hybridization. Molecular and Cellular Probes 22, 151-155.

Lopez-Flores, I., Robles, F., Valencia, J.M., Grau, A., Villalba, A., de la Herran, R., Garrido-Ramos, M.A., Ruiz-Rejon, C., Ruiz-Rejon, M., Navas, J.I., 2008b. Detection of Marteilia refringens using nested PCR and in situ hybridisation in Chamelea gallina from the Balearic Islands (Spain). Diseases of Aquatic Organisms 82, 79-87.

Marty, G.D., Bower, S.M., Clarke, K.R., Meyer, G., Lowe, G., Osborn, A.L., Chow, E.P., Hannah, H., Byrne, S., Sojonky, K., Robinson, J.H., 2006. Histopathology and a real-time PCR assay for detection of Bonamia ostreae in Ostrea edulis cultured in western Canada. Aquaculture 261, 33-42.

Montes, J., 1990. Development of Bonamia ostreae parasitosis of flat oyster, Ostrea edulis, from Galicia, Northwest Spain, Pathology in marine science. Proceedings of the Third International Colloquium on Pathology in Marine Aquaculture, held in Gloucester Point, Virginia, USA, October 2-6, 1988. Academic Press Inc., pp. 223-227.

OSPAR Convention for the protection of the marine environment of the North-East Atlantic, 2008. OSPAR Commission, London, UK.

Pascual, S., Villalba, A., Abollo, E., Garci, M., Gonzalez, A.F., Nombela, M., Posada, D., Guerra, A., 2010. The mussel Xenostrobus securis: a well-established alien invader in the Ria de Vigo (Spain, NE Atlantic). Biological Invasions 12, 2091-2103.

Journal Pre-proof

Journal Pre-proof

Perkins, F.O., Wolf, P.H., 1976. Fine-Structure of Marteilia-Sydneyi Sp-N - Haplosporidan Pathogen of Australian Oysters. Journal of Parasitology 62, 528-538.

Pichot, Y., Comps, M., Tige, G., Grizel, H., Rabouin, M.-A., 1981. Recherches sur Bonamia ostreae gen. n., sp. n., parasite nouveau de l'huitre plate Ostrea edulis. Studies on Bonamia ostreae gen. n., sp. n., a new parasite of the European flat oyster, Ostrea edulis L., Fr]. Rev Trav Inst Pêch Marit 43, 131-140.

Ramilo, A., González, M., Carballal, M.J., Darriba, S., Abollo, E., Villalba, A., 2014. Oyster parasites Bonamia ostreae and B. exitiosa co-occur in Galicia (NW Spain): spatial distribution and infection dynamics. Diseases of aquatic organisms 110, 123-133.

Ramilo, A., Navas, J.I., Villalba, A., Abollo, E., 2013. Species-specific diagnostic assays for Bonamia ostreae and B. exitiosa in European flat oyster Ostrea edulis: conventional, real-time and multiplex PCR. Diseases of Aquatic Organisms 104, 149-161.

Robert, M., Garcia, C., Chollet, B., Lopez-Flores, I., Ferrand, S., Francois, C., Joly, J.P., Arzul, I., 2009. Molecular detection and quantification of the protozoan Bonamia ostreae in the flat oyster, Ostrea edulis. Molecular and Cellular Probes 23, 264-271.

Ruiz, M., López, C., Lee, R.-S., Rodríguez, R., Darriba, S., 2016. A novel paramyxean parasite, Marteilia octospora n. sp.(Cercozoa) infecting the grooved razor shell clam Solen marginatus from Galicia (NW Spain). Journal of invertebrate pathology 135, 34-42.

Tige, G., Rabouin, M.-a., 1976. Etude d'un lot de moules transférées dans un centre touché par l'épizootie affectant l'huître plate. Proc. I.C.E.S K.

Van Banning, P., 1991. Observations on bonamiasis in the stock of the European flat oyster, Ostrea edulis, in the Netherlands, with special reference to the recent developments in Lake Grevelingen. Aquaculture 93, 205-211.

Villalba, A., Iglesias, D., Ramilo, A., Darriba, S., Parada, J.M., No, E., Abollo, E., Molares, J., Carballal, M.J., 2014. Cockle Cerastoderma edule fishery collapse in the Ria de Arousa (Galicia, NW Spain) associated with the protistan parasite Marteilia cochillia. Diseases of Aquatic Organisms 109, 55-80.

Journal Pre-proof

Villalba, A., Mourelle, S.G., Lopez, M.C., Carballal, M.J., Azevedo, C., 1993. Marteiliasis Affecting Cultured Mussels Mytilus-Galloprovincialis of Galicia (Nw Spain) .1. Etiology, Phases of the Infection, and Temporal and Spatial Variability in Prevalence. Diseases of Aquatic Organisms 16, 61-72.

Journal Pre-proof

Journal Pre-proof

Primers/Probes	Sequences	PCR product size
Mar-18S-F	5’ ACGATCAAAGTGAGCTCGTG 3’	
Mar-18S-R	5’ CAGTTCCCTCACCCCTGAT 3’	118 bp
Mar18S-IN (probe)	5’ GCATGGAATCGTGGAACGGG 3’ (FAM-BHQ-1)	
Bosp2-18S-F	5’ CAGGATGCCCTTAGATGCTC 3’	
Bosp2-18S-R	5’ GTACAAAGGGCAGGGACGTA 3’	199 bp
Bosp2-18S-IN (probe)	5’ TTGACCCGGCTTGACAAGGC 3’ (HEX-BHQ-1)	

Table 1: Sequences of the primers and probes of the new real-time PCR assay.

Host species	Status	Origin	PCR detection	
			M. refringens	***Bonamia* sp.**
Ostrea edulis	Not infected	France	No	No
Mytilus edulis	Not infected	France	No	No
Mytilus galloprovincialis	Not infected	France	No	No
Ostrea edulis	*M. refringens type M*	France	**yes**	No
Mytilus edulis	*M. refringens type M*	France	**yes**	No
Mytilus galloprovincialis	*M. refringens type M*	France	**yes**	No
Ostrea edulis	*M. refringens type O*	France	**yes**	No
Mytilus edulis	*M. refringens type O*	France	**yes**	No
Mytilus galloprovincialis	*M. refringens type O*	France	**yes**	No
Cerastoderma edule	*Marteilia cochillia*	Spain	**yes**	No
Saccostrea glomerata	*Marteilia sydneyi*	Australia	**yes**	No
Crassostrea gigas	*Marteilioïdes chungmuensis*	Japan	No	No
Ostrea edulis	*Bonamia ostreae*	France	No	**yes**
Ostrea edulis	*Bonamia exitiosa*	France	No	**yes**
Ostreae edulis	*Mikrocytos mackini*	Canada,	No	No
Crassostrea gigas	*Haplosporidium nelsoni*	France	No	No
Donax trunculus	*Mikrocytos donaxi*	France	No	No

Table 2: Analytical specificity. Samples infected with targeted parasites and other closely related parasites tested with the new real-time PCR assay.

	Marteilia refringens						***Bonamia sp.***					
Plasmidic DNA concentration (copies/µl)	**10^5**	**10^4**	**10^3**	**10^2**	**10**	**1**	**10^5**	**10^4**	**10^3**	**10^2**	**10**	**1**
% of positive results (N positive/ N tested)	**100% (24/24)**	**100% (24/24)**	**100% (24/24)**	**100% (24/24)**	**96% (23/24)**	**21% (5/24)**	**100% (24/24)**	**100% (24/24)**	**100% (24/24)**	**100% (24/24)**	**96% (23/24)**	**25% (6/24)**
Mean Ct value (n=24)	**24.71**	**28.31**	**31.53**	**34.95**	**37.81**	**NA**	**22.01**	**25.62**	**29.42**	**33.03**	**36.86**	**NA**
Intra assay standard deviation	0.93	0.71	1.24	1.33	0.59	NA	0.27	0.51	0.86	0.84	1.00	NA
Inter assay standard deviation	0.00	0.44	0.00	0.80	0.91	NA	0.65	0.55	0.37	0.29	0.54	NA
Intermediate precision (IP)	0.93	0.83	1.24	1.55	1.08	NA	0.70	0.75	0.93	0.89	1.14	NA
Coefficient of variation of IP (%)	3.77	2.95	3.93	4.44	2.87	NA	3.19	2.93	3.17	2.70	3.08	NA

Journal Pre-proof

Journal Pre-proof

Table 3: Analytical sensitivity, repeatability and preliminary reproducibility. 10-fold serial dilutions of plasmidic DNA were tested in 8 replicates x 3 PCR runs (N=24 PCR tests for each dilution). Repeatability and preliminary reproducibility were assed based on the Ct values of the 24 replicates tested for each dilution. Parameters were calculated according to ISO 5725-2.

4.A

Marteilia refringens

	New PCR	Conv.PCR	N
"High" prevalence group (N=51)	-	-	10
	-	+	1
	+	-	12
	+	+	28
"Low" prevalence group (N=235)	-	-	215
	-	+	0
	+	-	11
	+	+	9
"Parasite free" group (N=100)	-	-	100
	-	+	0
	+	-	0
	+	+	0

Bonamia ostreae

	New PCR	Conv. PCR	N
"High" prevalence group (N=45)	-	-	18
	-	+	3
	+	-	0
	+	+	24
"Low" prevalence group (N=101)	-	-	88
	-	+	0
	+	-	2
	+	+	11
"Very low" group (N=203)	-	-	198
	-	+	2
	+	-	2
	+	+	1

4.B

	M. refringens DSe	*M. refringens* DSp	*B. ostreae* DSe	*B. ostreae* DSp
New PCR	87.2% [70.9-99.4]	98.4% [95.8-99.9]	77.5% [55.1-96.0]	98.4% [96.6-99.6]
Conv. PCR	60.7% [44.6-78.3]	99.7% [99.8-99.9]	82.8% [60.1-97.8]	98.7% [97.1-99.7]

Table 4: 4A - Results obtained with the new real-time PCR and conventional PCR on three groups of flat oysters collected on the field, in B. ostreae *and / or* M. refringens *endemic areas. Left: Results for the detection of* Marteilia refringens *with the new real-time PCR and the conventional PCR developed by Le Roux et al. 2001 (Le Roux et al., 2001) Right: Results for the detection of* Bonamia sp *with the new real-time PCR and the conventional PCR developed by Cochennec et al.2000.*

Journal Pre-proof

4B - Diagnostic sensitivity (DSe) and specificity (DSp) of the new real-time PCR and conventional PCR assays estimated by Bayesian latent class analysis. 95% credibility intervals are indicated in brackets.

Bonamia exitiosa		
New PCR	Conv. PCR	N
-	-	26
-	+	2
+	-	1
+	+	3

Table 5: Results obtained with the new real-time PCR and conventional PCR (Cochennec et al. 2000) (Cochennec et al., 2000) for the detection of Bonamia *sp. on the* B. exitiosa *infected group.*

	M. refringens **detection**	***Bonamia*** **sp. detection**
Average % good results	98.3%	96.1%
Sensitivity	99.3%	99.3%
Rate of false negatives	0.7%	0.7%
Specificity	97.8%	94.5%
Rate of false positives	2.2%	5.5%

Table 6: Synthetic results of the Inter Laboratory comparison study based on results obtained by the 17 participating laboratories on a set of 24 samples (8 negative samples, 8 Marteilia refringens *positive samples, and 8* Bonamia ostreae *positive samples) using the new real-time PCR. Sensitivity is the proportion of samples from known infected samples that tested positive with the new PCR. Rate of false negatives= 1-Se. Specificity is the proportion of samples from known uninfected samples that tested negative with the new PCR. Rate of false positives = 1-Sp.*

	Detection of *Marteilia refringens*		
Samples	Accordance % (within laboratory variation)	Concordance % (between laboratory variation)	Concordance Odds Ratio (COR)
Non infected with *Marteilia refringens*	97.3	95.5	1.67

Journal Pre-proof

Infected with *Marteilia refringens*	99.0	98.0	1.00
Global	**97.9**	**96.4**	**1.72**

	Detection of *Bonamia* sp.		
Samples	Accordance % (within laboratory variation)	Concordance % (between laboratory variation)	Concordance Odds Ratio (COR)
Non infected with *Bonamia* sp.	94.2	89.3	1.94
Infected with *Bonamia* sp.	99.1	98.7	1.51
Global	**96.1**	**93.3**	**1.76**

Table 7: Method precision: Accordance, Concordance and Concordance Odds Ratio based on the results of 17 laboratories that tested a set of 24 samples of flats oysters with the new real-time PCR.

Journal Pre-proof