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NOTE

Detection and characterization of *Bonamia ostreae* in *Ostrea edulis* imported to China

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ABSTRACT: The protozoan parasite *Bonamia ostreae* is a destructive pathogen of flat oysters and has been reported to be widespread in Europe and North America. The biological characteristics of this unicellular parasite are still not fully understood. In this study, 104 *Ostrea edulis* imported from the USA to the Guangdong province of China for consumption were examined for *Bonamia* infection. PCR assay, combined with restriction fragment length polymorphism, sequencing and BLAST analysis, showed that *B. ostreae* DNA could be detected in 1 of the 104 oyster samples. Light microscopy revealed *Bonamia*-like organisms in the oyster. PCR assay and fluorescent *in situ* hybridization showed that *B. ostreae* organisms were present and retained their integrity after 4 wk in culture. Acridine orange-ethidium bromide staining indicated that the *B. ostreae* were still alive. In conclusion, *B. ostreae* was present in oysters imported to China. More importantly, the parasite was able to survive for at least 4 wk of *in vitro* culture at 4°C, which further implied a long-term transmission risk of *B. ostreae*. Considering the wide culture beds of *Crassostrea ariakensis* and *C. gigas* in China, and that *C. ariakensis* and *C. gigas* are susceptible hosts or reservoirs of *B. ostreae,* our study highlights the potential risk of introducing *B. ostreae* by importing *O. edulis* from a *Bonamia* endemic area.

KEY WORDS: *Bonamia ostreae* · *Ostrea edulis* · *In vitro* culture characterization · Fluorescent *in situ* hybridization · Transmission risk

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INTRODUCTION

Bonamia ostreae is an intracellular protozoan parasite that infects the haemocyte of flat oysters. During infection, extracellular forms can also be found among the epithelial or interstitial cells in the gill, lobe, heart tissue, stomach or connective tissue of the oysters (Culloty &Mulcahy 2007). These parasites are unicellular organisms with a diameter of 1 to 3 µm, also known as 'microcells' (Carnegie & Cochennec-Laureau 2004). *Bonamia ostreae*, together with *B. exitiosa*, *B. roughleyi* and *B. perspora*, belong to the order Haplosporidia, within the phylum Cercozoa (Brehélin et al. 1982, Carnegie et al. 2000). Among these species, *B. ostreae* and *B. exitiosa* are the cause of serious internationally notifiable diseases (World Organisation for Animal Health [OIE] 2012).

Since its first identification in California (Elston et al. 1986), *Bonamia ostreae* has spread to Maine and Washington in North America and to France and Spain in Europe (Elston et al. 1986, Zabaleta & Barber 1996, Cigarría & Elston 1997). Furthermore, *B. ostreae* has also been found in many countries such as England, Italy, Ireland and the Netherlands, where production of flat oysters occurs (Bannister & Key 1982, Figueras 1991, McArdle et al. 1991, Engelsma et al. 2010, Narcisi et al. 2010). As oysters are reared in the open area of the sea, it is impossible

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to develop effective methods to treat bonamiosis. Thus, preventing the transmission of the pathogen from infected areas to non-infected areas seems to be one of the most effective control strategies.

Several flat oyster species are known to be susceptible to *Bonamia ostreae*, for example, *Ostrea edulis*, *O. angasi, O. puelchana* and *Crassostrea ariakensis* (Carnegie & Cochennec 2004, Audemard et al. 2005, OIE 2012). *C. gigas* may be a carrier or reservoir of *B. ostreae* and *B. exitiosa* (Lynch et al. 2010). Translocation of the above infected live hosts is recognized as a major underlying cause of the spread of bonamiosis. Considering the importance of the commercial exchange of live oyster stocks around the world and the associated risk of spreading the disease from infected to non-infected areas, many detection techniques have been developed. These techniques include microscopic observation, conventional PCR combined with restriction fragment length polymorphism (RFLP) analysis, real-time quantitative PCR (qPCR), *in situ* hybridization or fluorescent *in situ* hybridization (FISH) and electrochemical genosensors (Berthe et al. 1999, Carnegie et al. 2000, 2003, Cochennec et al. 2000, da Silva & Villalba 2004, Corbeil et al. 2006, Narcisi et al. 2011). Among these methods, FISH is a powerful complement to morphological and PCR-based detection methods. PCR (using the genus-specific primers Bo-Boas), and RFLP analysis is the method recommended by the World Organisation for Animal Health (OIE) for identification of *B. ostreae*.

The biological characteristics of *Bonamia* sp., such as transmission, life cycle and *in vitro* survival ability, are still not fully understood. In this study, we examined 104 *Ostrea edulis* samples imported to China directly for consumption for the presence of *B. ostreae*. The infected material was used for further characterization of the pathogen. Considering the potential serious consequences of introducing the pathogen to China, the *in vitro* survival time of *B. ostrea*e was studied to assess the introduction risk.

MATERIALS AND METHODS

Sample collection and DNA extraction

A total of 104 samples of *Ostrea edulis*, imported from the USA to China for consumption, were collected by the Guangdong Entry–Exit Inspection and Quarantine Bureau, PR China, in December 2011 and July 2012. DNA extraction was performed as described by Cochennec et al. (2000). After oyster shucking, gill tissue (50 mg) was collected and DNA was extracted using a QiaAmp DNA Mini-kit (Qiagen), according to the manufacturer's instructions. DNA was eluted into 50 µl of sterile, deionized water and stored at −20°C prior to PCR.

PCR-RFLP identification

PCR was used as the primary *Bonamia ostreae* screening method, as recommended by the OIE (2012). The reaction was expected to amplify a DNA fragment of approximately 300 bp. The PCR primers were as follows: Bo: 5′-CAT TTA ATT GGT CGG GCC GC-3′, Boas: 5′-GGG GGA TCG AAG ACG ATC AG-3′. The 25 µl PCR mixtures contained 2.5 µl 10× *Taq* buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 µM Bo/Boas primers, 2.5 U r*Taq* polymerase (TaKaRa) and 2 µl DNA templates. The PCR reaction mixtures were denatured at 94°C in a thermocycler for 5 min before being submitted to 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Subsequently, RFLP was performed. *Hae*II and *Bgl*I were used to digest the Bo-Boas amplicons. Then, 5 µl digested products were analyzed by electrophoresis on agarose gels, stained with SYBR-Green (Invitrogen) and visualized under UV light.

Light microscopic observation

A small piece of fresh gill tissue from the PCR-positive sample was sliced and fixed in Davidson's solution for 48 h. Gill tissue from a PCR-negative sample was processed in the same way and used as a negative control. After dehydration, the tissues were embedded in paraffin. The paraffin blocks were then cut into 5 µm thick sections. Paraffin sections from the same tissue block were stained with haematoxylin and eosin (H&E) (Kim et al. 2006) or diamidino-2-phenylindole (DAPI), a fluorescent stain binding strongly to AT-rich regions in DNA. Subsequently, these sections were observed under an Olympus DP72 fluorescence microscope.

In vitro survival time and purification

To assess the potential risk of introducing *Bonamia ostreae* through importing oysters, the *in vitro* survival time of the parasite in the infected *Ostrea edulis* tissue was investigated. Whole body tissue of

O. edulis was rinsed 3 times in sterile seawater and then decontaminated twice for 30 min in 30 ml sterile seawater with an antimicrobial suspension (61.3 mg l^{-1} penicillin G, 131 mg l^{-1} streptomycin sulphate, 50 000 U l^{-1} nystatin). The tissue was rinsed again and 4 ml sterile seawater was added per 10 g of tissue. The tissue was cut into small pieces and homogenized for 10 s at 10 s intervals in a 50 ml amicrobic tube using a GINGKO G20 Motor Driven Tissue Grinder until no obvious tissue pieces could be seen. The homogenate was then stored at 4°C. Morphological observation of the cultivated parasites was performed under a light microscope. DNA extraction and PCR (using the Bo-Boas primers described above) was performed on a 0.5 ml cultivation mixture once a week. After confirmation of infection status by PCR, 10 *B. ostreae*-free *Crassostrea ariakensis* collected from the Guangdong province were homogenized using the method described above and used as a control.

After cultivation at 4°C for 4 wk, the parasites were purified on sucrose gradients and then a Percoll gradient, following the method described by Mialhe et al. (1988). The 50 to 60 % and 60 to 70 % interface layers were collected and re-suspended in 100 µl of seawater prior to FISH.

Fluorescent *in situ* hybridization and viability

The probe Bo-1 (5′-TCT GGC CCG GCG ATA CTA GCA CCC-3′-FITC), corresponding to a fragment inside the Bo-Boas amplicon and labelled with fluorescein isothiocyanate (FITC), was designed based on the consensus rDNA sequences of *Bonamia ostreae*. BLAST analysis of the probe revealed no cross matches; thus, it should be specific for *B. ostreae*. FISH was performed as described previously (Carnegie et al. 2003, Arzul et al. 2012) with some modifications. Purified parasites were fixed on silane-prep slides (Sigma), and the slides were then treated with pronase (20 µg ml^{-1} in double-distilled water [ddH_2O]) at 37°C for 10 min. After pre-hybridization and hybridization (with 10 ng $µl^{-1}$ probe or FITC) at 42°C overnight, the samples were washed 3 times in phosphate-buffered saline (PBS) with 0.1 % Triton X-100 at 42°C. Percoll gradient-purified parasite hybridized with FITC (Sigma) was chosen as a non-specific negative control. A healthy *Ostrea edulis* gill tissue section hybridized with the Bo-1 probe was used as a tissue control. Photographs of the FISH analysis were taken with the same fluorescence exposure time, using an Olympus DP72 fluorescence microscope.

Cell viability was determined using acridine orange-ethidium bromide staining, as described by Mialhe et al. (1988). In this assay, live cells appear green while dead cells appear orange.

RESULTS

Detection of *Bonamia* sp. in *Ostrea edulis*

Among the 104 collected oysters, 1 was suspected to be *Bonamia*-positive as PCR analysis resulted in a 300 bp product (Fig. 1). Sequencing and BLAST analysis of the 300 bp PCR product indicated that this fragment had 100 % identity with the rDNA of *B. ostreae* from the USA (AF262995) and France (AF192759). Subsequent RFLP analysis was carried out on the 300 bp amplicon, using the endonucleases *Hae*II and *Bgl*I. The results showed that the 300 bp amplicon could be digested by both enzymes (Fig. 1). Bands of approximately 110 and 190 bp were obtained by digestion with *Hae*II, and of approximately 120 and 180 bp by digestion with *Bgl*I.

Histological observation

Histological examination by H&E staining showed high numbers of *Bonamia*-like organisms in the gill tissues of the infected oyster. In the non-infected con-

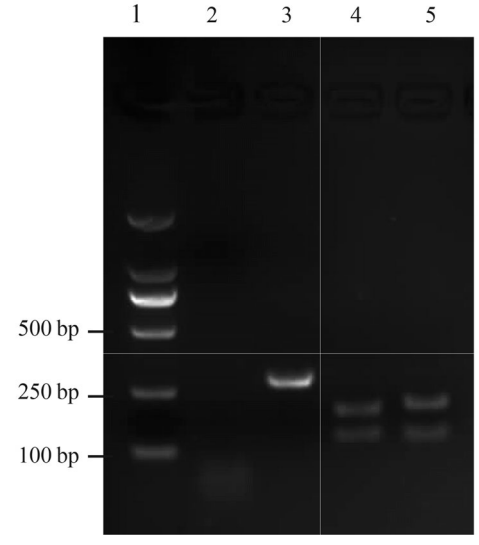

Fig. 1. *Bonamia ostreae* in *Ostrea edulis*. Agarose gel electrophoresis of Bo-Boas PCR amplicons. Lane 1: marker; Lane 2: negative control; Lane 3: PCR products (by Bo-Boas) from the positive infected sample; Lane 4: enzyme digestion of the amplicon by *Bgl*I; lane 5: enzyme digestion of the amplicon by *Hae*II

trol, the host cells were usually basophilic (Fig. 2A). In the infected gill tissue, the *Ostrea edulis* cells and the uninucleated organisms were stained different colours by H&E. The host cells were still basophilic, as in the non-infected control, while the uninucleated organisms inside the host haemocytes were eosinophilic (Fig. 2B). The uninucleated organisms were clustered in the host haemocyte or present as extracellular free particles, which were spherical or ovoid and approximately 2 µm in diameter. As shown in Fig. 2C,D, the uninucleated organisms were stained blue by DAPI, indicating that they contain DNA and are microcells rather than epithelial eosinophilic granulocytes.

In vitro survival time of *Bonamia ostreae*

As an important biological characteristic, the *in vitro* survival time of *Bonamia ostreae* inside host homogenate was further investigated. PCR examination of the cultured mixture was carried out once a week. Results showed that the 300 bp product could be amplified from the culture after storage at 4°C for 4 wk. In contrast, no PCR product was amplified from the non-infected control (Fig. 3).

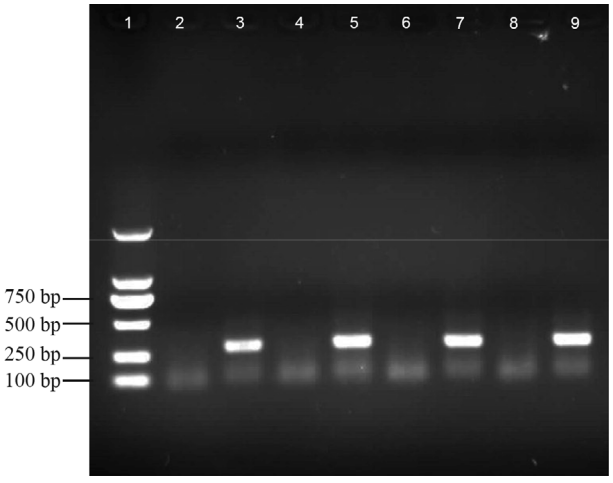

Fig. 3. *Bonamia ostreae* in *Ostrea edulis*. Weekly PCR detection of *B. ostreae* from the culture mixture. Lane 1: marker; Lane 2: PCR amplification from the negative control after 1 wk; Lane 3: PCR amplification from the *B. ostreae* culture mixture after 1 wk; Lane 4: the negative control after 2 wk; Lane 5: the *B. ostreae* culture mixture after 2 wk; Lane 6: the negative control after 3 wk; Lane 7: the *B. ostreae* culture mixture after 3 wk; Lane 8: the negative control after 4 wk; Lane 9: the *B. ostreae* culture mixture after 4 wk

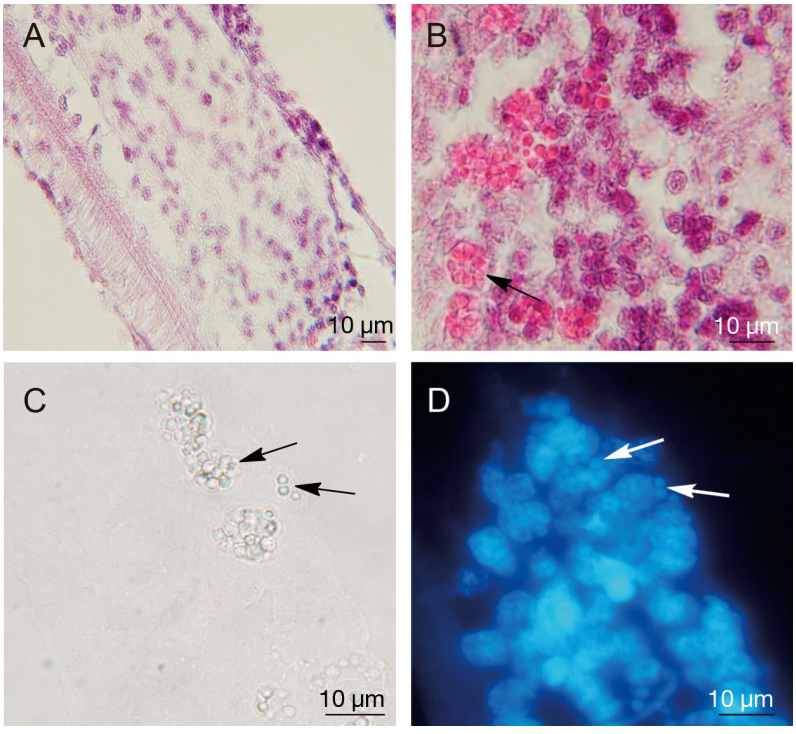

Fig. 2. *Bonamia ostreae* in *Ostrea edulis*. Histological observation of uninucleated organisms in gills. Haematoxylin and eosin (H&E) staining of gill tissue from (A) healthy and (B) infected *O. edulis*. Arrow indicates a cluster of uninucleated organisms. (C,D) Another section from the same tissue block stained with DAPI; (C) shown under bright field and (D) the same view with DAPI-stained DNA fluorescing blue under UV light excitation. Arrows indicate uninucleated organisms, as in (B). Blue staining in (D) proves these cells contain DNA and are not host cell organelles

However, the PCR results could only demonstrate the presence of the DNA, not the living organism. The FISH assay is a powerful tool for the detection *Bonamia ostreae* (Carnegie et al. 2003) that can identify the pathogen both molecularly and histologically with little non-specific stain. Fig. 4A illustrates the autofluorescent background in the absence of probe in our FISH experiment. Only dark green autofluorescence and the black background could be observed. Fig. 4B indicates that no cross reaction between the probe Bo-1 and host tissue occurred. Fig. 4C shows that *B. ostreae* could be detected by the specific probe Bo-1 and were stained green. Most of the *B. ostreae* were clustered together, while some of them were free. The cells were approximately 1 to 2 µm, which is within the typical size range of *B. ostreae*. The morphol-

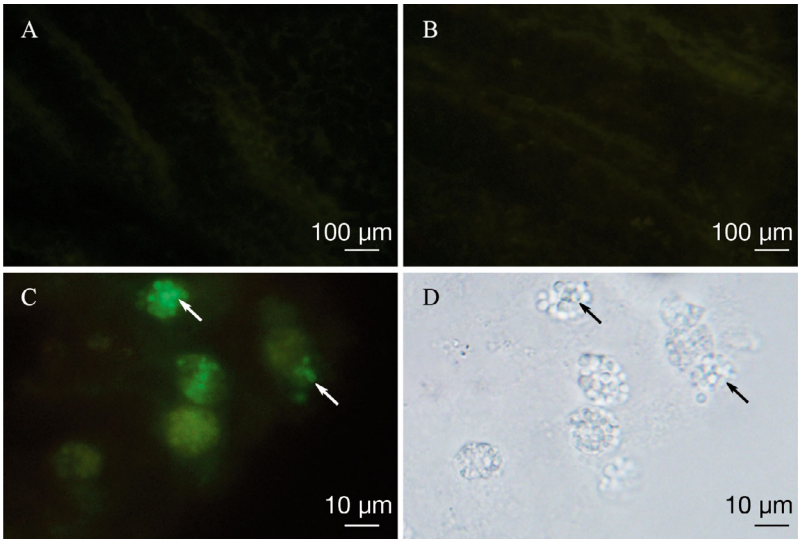

Fig. 4. *Bonamia ostreae* in *Ostrea edulis*. Fluorescent *in situ* hybridization (FISH) trials. (A) Control using FITC hybridized with purified parasites. The specific probe Bo-1 hybridized with (B) a healthy *O. edulis* gill tissue section or (C) with purified parasites. Arrows in (C) indicate clustered or single *B. ostreae* cells. (D) The same *B. ostreae* cells under light field. Arrows indicate the cell clusters

ogy of corresponding cells observed under light field was similar to that of the *B. ostreae* cells previously stained with H&E (Fig. 4D).

To verify the viability of *Bonamia ostreae*, the cells were directly observed in light field and under fluorescence after staining with acridine orange and ethidium bromide. In light field, some cells showed the typical morphological characteristics of *B. ostreae*, including the round shape, 'fried egg' appearance, 1 to 2 µm diameter and clustering together (Fig. 5A). These cells showed obvious integrity. Under fluorescence, some of these cells appeared green, which indicated that they were alive (Fig. 5B).

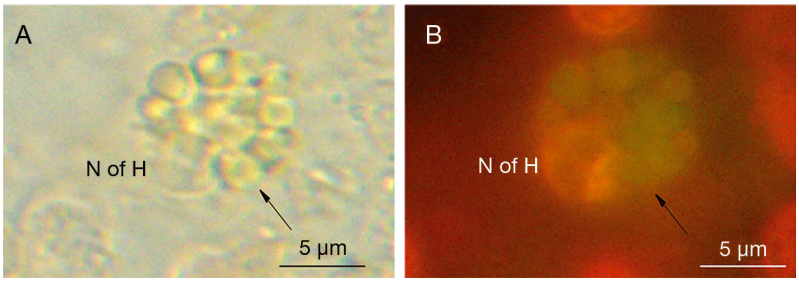

Fig. 5. *Bonamia ostreae* in *Ostrea edulis*. Detection of cell viability by acridine orange and ethidium bromide staining. (A) Cultured cells under light field. *B. ostreae* (arrow) are clustered near the nucleus of a host haemocyte (N of H). (B) Cultured cells under fluorescent field. *B. ostreae* cells are green and indicated by an arrow

DISCUSSION

Bonamia ostreae is an economically significant parasite of oysters and was originally enzootic in Europe and North America (Culloty & Mulcahy 2007). Bonamiosis is not only notifiable to the OIE, but is also listed as a 'Class C' disease and notifiable to the Ministry of Agriculture of China. Prior to this study, no official report of this disease had been recorded in Asian countries, including China, according to the OIE (www.oie.int/wahi2s_2/public/wahid.php/Diseaseinformation/WI#) and the official veterinary bulletin of the Ministry of Agriculture of China (www.moa.gov.cn/zwllm/tzgg/gb/sygb/). PCR-RFLP analysis of *Crassostrea gigas* (a potential carrier) and *C. ariakensis* (a susceptible host) from the Chinese coast, including the Bohai Sea, Yellow Sea and East China Sea, found them to be negative for *Bonamia* sp. infection (our previous epidemiological investigation in 2011, unpubl. data). It is very important to prevent the spread of this pathogen to China by developing reliable quarantine methods and paying special attention to transmission routes and the potential role of reservoir hosts. In this study, the *in vitro* survival time of *B. ostreae* was investigated to further assess the risk of introduction from imported oysters.

Our PCR and subsequent sequence analysis results confirmed that *Bonamia ostreae* DNA was present in 1 of the 104 imported oysters (Fig. 1). Furthermore, judging by morphological characteristics, *B. ostreae*-like uninucleated organisms were present in infected tissue (Fig. 2). Given that the oyster samples were imported for consumption and never removed from their travel packaging until arrival in our laboratory, it is almost certain that this oyster was infected before shipment.

Our investigation showed that *Bonamia ostreae* can survive *in vitro* at 4°C, and retain typical cell morphology (Figs. 3, 4 & 5). Temperature has been found to be one of the most important factors affecting the survival time of *B. ostreae in vitro*. Culloty & Mulcahy (2007) demonstrated

that lower temperatures are associated with increased bonamiosis prevalence. Arzul et al. (2009) proved that *B. ostreae* showed a significantly higher survival rate at 4°C compared to that at 15 and 25°C. The survival rate of the pathogen after 2 d at 4°C ranged from 52 to 74%, while at the higher temperature of 25°C, this was reduced to 22–53%. It has also been reported that low temperature can affect the defensive capabilities of the phagocytic haemocytes of flat oysters (Gagnaire et al. 2006), enabling the parasite to survive longer. Imported oysters are always maintained at a low temperature or on ice to keep them fresh during transportation and sale, which offers suitable conditions for the survival of *B. ostreae* and further increases the risk of introduction.

Bonamia sp. can be spread by reuse of contaminated equipment or by fouling on boat hulls in infected areas (Cigarría & Elston 1997). Both of these could explain the presence of this parasite near a port in the eastern USA and in Spain (Friedman& Perkins 1994, Cigarría & Elston 1997, Abollo et al. 2008). However, biological characteristics of the pathogen such as *in vitro* survival time, which is a very important characteristic related to transmission, have not been well studied. Interestingly, according to our results, *B. ostreae* can survive for as long as 4 wk *in vitro*. This is significantly longer than the previously reported survival time of 1 wk in filtered seawater (Arzul et al. 2009), or 2 wk in another study (Grizel 1985). More importantly, Guangdong province acts as a major production area of *Crassostrea ariakensis* and *C. gigas* in China (Wang 2004), and these may be the susceptible hosts or carriers of *B. ostreae* (Lynch et al. 2010, OIE 2012). If *B. ostreae* spreads to the culture beds of these oysters during its survival time, this may result in high prevalence. However, parasite survival for 4 wk under laboratory conditions may not be relevant to their survival in the wild. More experiments to investigate the survival time of *B. ostreae* in the wild need to be carried out.

In conclusion, this study confirmed the presence of *Bonamia ostreae* in imported *Ostrea edulis.* Our results suggest that, under the suitable conditions, *B. ostreae* might be able to survive in dead oysters for 4 wk. In the absence of proper quarantine, unsuitable disposal of dead infected *O. edulis* may potentially result in the release of viable infective pathogen into the sea and cause infection and disease. It is hoped that these results will contribute to a better understanding of the basic biology of this parasite as well as of the host–parasite relationship.

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