Aquat. Living Resour. 23, 125–130 (2010)

DOI: 10.1051/alr/2010003
www.alr-journal.org

Aquatic Living Resources

Note

Use of histopathology, PCR and in situ hybridization methods to detect the parasite *Mikrocytos* sp. in Pacific oyster *Crassostrea gigas* from the northern coast of the Yellow Sea, China

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Received 21 May 2009; Accepted 19 November 2009

Abstract – *Mikrocytos mackini* is the etiological agent of Denman Island disease, which causes significant mortalities in commercially important bivalve species, including the Pacific oyster, *Crassostrea gigas*. A close relative of *M. mackini*, *Mikrocytos* sp., was recently detected in oysters imported into France from Canada. In this study, we examined Pacific oysters from the northern coast of the Yellow Sea, China. Of the one hundred samples examined histologically, a microcell parasite was found in the tissues of four oysters. To identify whether the parasite was *Mikrocytos* sp., DNA was extracted from the oysters and polymerase chain reaction (PCR) amplifications were performed with primers (Mikrocytos-F and Mikrocytos-R), which yielded the expected 522 bp fragment. DNA sequencing of these products confirmed that they were identical to the corresponding 18S region of *Mikrocytos* sp. (100%) and had close similarity to *M. mackini* (89%). In situ hybridization (ISH) also was performed in this study, and the primer pair MM-like (CCTGTCCTATGTCGGGCAGG) hybridized with the Pacific oyster parasite. This is the first report of *Mikrocytos* sp. in the Pacific oyster from the coast of China. Although this study suggests a low prevalence of the parasite in China, its potential threat to aquaculture should be considered.

Key words: Parasite / *Mikrocytos mackini* / *Mikrocytos* sp. / Histology / Polymerase chain reaction / In situ hybridization / *Crassostrea gigas*

1 Introduction

There are more than twenty species of oysters present along the coast of China. Among these, *Crassostrea rivularis*, *Crassostrea gigas* and *Saccostrea cucullata* are regarded as the most suitable species for culture and have been widely cultivated for many years. The Pacific oyster, *C. gigas*, mainly exists along the coast of the Bohai and the northern coasts of the Yellow Sea. Originating from northeastern Asia, *C. gigas* is endemic to Japan, but has been introduced and translocated, mainly for aquaculture purposes, to several other areas, including the north west Pacific and the Atlantic coasts of France, Spain, Portugal, and Morocco (CIESM 2000). *C. gigas* was imported into China from Japan in the 1980s and has been cultured on a large scale in Liaoning and Shandong provinces. The Pacific oyster has been a major economic resource for many people living along the coast in these areas. In 2004, China was the world leader in *C. gigas* production, with 3.75 out of a total world production of 4.6 million tonnes (81% of world production) (Lapègue et al. 2007). Since 1994, however, mortality (70–80%) in 2-year-old *C. gigas* has been reported in summer for certain coastal areas of Dalian and Shandong Province (Ma et al. 1997; Sui et al. 2002).

Oyster diseases mostly are caused by viruses, bacteria, and endoparasites (Bower et al. 1994). Of these, endoparasitic protozoans are the most harmful pathogens in oyster culture and have been widely studied. To date, the protozoan parasites *Haplosporidium nelsoni* (MSX) and *Haplosporidium costale* (SSO) have been detected in *C. gigas* in China (unpublished data). Species of the protozoan *Mikrocytos* represent one of the most serious causes of oyster mortality worldwide. Mikrocytosis is caused by the parasite *Mikrocytos mackini*. In several studies in Canada, mikrocytosis caused about 30% mortality in market-sized *C. gigas* in spring (Quayle 1961; Bower 1988). Susceptible host species of *M. mackini* include *C. gigas*, *Crassostrea virginica* (Eastern oyster), *Ostrea edulis* (European flat oyster), and *Ostrea conchaphila*

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(Olympia oyster) (Bower et al. 1997; 2005). *M. mackini* is on the World organisation for animal health (OIE) list of diseases in mollusc. According to OIE, *M. mackini* is distributed along the Canadian west coast, is probably ubiquitous throughout the Strait of Georgia, and is found in other specific localities around Vancouver Island and adjacent areas of the state of Washington, USA. Susceptible species held at < 10 °C for at least 3 months are vulnerable to *M. mackini* infection (Hervio et al. 1996; Bower et al. 1997). *M. mackini* infection is prevalent in April and May, and infection can be fatal depending on the host and environmental conditions (Bower 1988, 2001). Recently, an unidentified microcell parasite believed to be a *Mikrocytos*-like parasite was detected in *O. edulis* from Canada after transport and quarantine in France (Gagné et al. 2008). In that study, *Mikrocytos* sp. was diagnosed by histology, heart smears, polymerase chain reaction (PCR), and in situ hybridization (ISH). To date, neither *M. mackini* nor *Mikrocytos* sp. have been reported in Asia (OIE 2006). Because Pacific oysters are an important commercial resource in China, understanding the protozoan diseases that are present in this species is important. Thus, the aim of this study was to assess Pacific oysters from the northern coast of the Yellow Sea, China, for the presence of *Mikrocytos* sp. using histology, PCR, and ISH.

2 Materials and methods

2.1 Site location, holding conditions, and monitoring

One hundred cultured *C. gigas* were collected for routine histological surveys from two locations along the northern coast of the Yellow Sea of China between January 2007 and May 2007. The oysters had been produced in a hatchery and transferred to open water after three months. They were reared in suspended culture in an open water intertidal area and were 3 years old when they were sampled and taken to our laboratory. In January 2007, fifty *C. gigas* were collected from Dayaowan Bay, Dalian (Liaoning Province) to search for the presence of *Mikrocytos* using histology. Subsequently, after *Mikrocytos* sp. was found in Dalian, fifty oysters from Changdao (Shandong Province) were collected in May 2007. The aim was then to determine whether this parasite was also present in Changdao.

Fifty oysters were examined at each location and shell length of each oyster was recorded. Epibiota were removed to prevent contamination (Littlewood and Ford 1990). Between oyster dissections, scalpels were soaked in hypochlorite and deionized water to prevent contamination between oysters. Date, location, and shell length were noted (Table 1).

Table 1. Data collected, site, and shell length of *C. gigas* samples.

Sampling date	Sampling site	Sampling location	Shell length (mm)	Salinity and Temperature
01.24.2007	Dayaowan Bay	121°54′04.0″	84.0	29.2‰,
07		39°01′30.3″	82.4	2.5 °C
05.31.2007	Changdao	120°42′40.0″	88.5	29.0‰,
07		37°56′55.3″	70.0	15.5 °C

2.2 Diagnostic methods

Histology

A conventional histological preparation was performed. Half of the tissue of each oyster (including gill, gonad, adductor muscle and digestive gland) was fixed in Bouin's solution with acetic acid, embedded in paraffin, sectioned (4 μm thickness), and stained with hematoxylin and eosin. A complete examination of the section was performed under a light microscope. The other half of the soft tissue of each oyster was used for genomic DNA extraction and PCR analysis.

DNA extraction and PCR

Genomic DNA was extracted from 50 mg of tissue using the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. All DNA samples were stored in TE buffer at –20 °C.

DNA was used for PCR amplification using the primers Mikrocytos-F (AGATGGTTAATGAGCCTCC), Mikrocytos-R (GCGAGGTGCCACAAGGC) (Carnegie et al. 2003), and Mmack (CATTGGAGGAGTCAGAGGGTG) (Gagné et al. 2008). Reactions were performed in a 50 μl volume containing 5 μl of 10 × PCR buffer (Mg^{2+}; 100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$), 4 μl dNTPmix (2.5 mM each of dATP, dCTP, dGTP, dTTP), 0.25 μl TaKaRa EX Taq™ (5 U μl^{-1}), 0.5 μl of each primer (20 pmol μl^{-1}), and 4 μl of template DNA. Reaction mixtures were cycled in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, USA). The program used was as follows: initial denaturation at 94 °C for 10 min; 30 cycles at 94 °C for 1 min, 60.5 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. PCR amplification products were separated by electrophoresis on a 2% agarose gel and GeneFinder staining (Bio-V, China) before being sequenced by the TaKaRa Biotechnology Company (Dalian, China). DNA from an uninfected oyster was used as a negative control. Positive control DNA from oysters confirmed to contain *H. nelsoni* and *H. costale* (provided by this laboratory) was used in the respective assays.

DNA sequencing

The PCR products were purified and cloned by the TaKaRa Biotechnology Company (Dalian, China). A recombinant plasmid from each PCR product was sequenced using a ABI PRISM™ 3730 XL DNA Analyzer (Applied Biosystems) using the BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Sequences were verified using BLAST searches of the GenBank database (http://www.ncbi.nlm.nih.gov).

In situ hybridization (ISH)

To identify the parasite found in histological sections, ISH was performed on the digestive gland tissue sections of the oysters that were found to contain *Mikrocytos* sp in the histological examination. The specific primer pair MM-like (CCTGTCCTATGTCGGGCAGG) (Gagné et al. 2008) was used to produce a digoxigenin-labeled probe by PCR at the TaKaRa Biotechnology Company (Dalian, China). PCR amplification was performed as above except that 2 μl of DIG dUTP (20 mM) were added to the reaction mixtures. This *Mikrocytos* probe was applied to ISH on paraffin-embedded tissue sections following the procedures described by Meyer et al. (2005) and Gagné et al. (2008). Briefly, tissue sections adhering to silane-coated slides were deparaffinized in xylene (2 × 5 min), rehydrated through an ethanol series (2 × 100, 90, 80, 70, 60, 50, and 30% for 2 min each), washed for 1 min in tap water, and equilibrated in phosphate-buffered saline (PBS) (3 × 5 min). Proteinase K (0.1 mg ml^{-1}) in PBS was applied for 15 min at 37 °C. Proteolysis was halted with a wash in PBS containing 4% paraformaldehyde and 0.2% glycine for 10 min at room temperature. The sections were washed for 5 min in PBS before being acetylated using acetic anhydride (0.2%) in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Slides were washed for 5 min in PBS and dried for 10 min in air. Sections were incubated with 20–30 μl of prehybridization buffer (5 ×SSC, 50% formamide, 5 × Denhardt's solution, 0.5% sodium dodecyl sulphate (SDS), 0.1 mg ml^{-1} denatured herring sperm DNA, 10% dextran sulfate) for 1–2 h at 42 °C, then the solution was replaced with 20–30 μl hybridization buffer containing 20 ng ml^{-1} of the digoxigenin-labeled MM-like probe. The sections were covered with plastic cover slips and placed on a heated block at 95 °C for 5 min, then cooled on ice for 10 min and incubated at 42 °C overnight in a humid chamber. The sections were washed twice for 5 min in 2 × SSC at room temperature and twice for 15 min in 0.1 × SSC at 42 °C. Sections were washed in buffer containing 1 × maletic acid and 0.3% (v/v) Tween-20 for 10 min. Following this step, detection was conducted using the Dig Probe detection kit (Innogent Biotechnology Company, Shenzhen, China) according to the manufacturer's instructions. Uninfected *C. gigas* was tested by ISH as a negative control. In addition, the DIG-ISH probe was used on tissue sections from *C. gigas* that had been confirmed to contain the parasite by histology, using the probes MS X 1347 (specific for *H. nelsoni*) and SSO 1318 (specific for *H. costale*) (Stokes and Burreson 1995) (provided by this laboratory).

3 Results

3.1 Histology

Observation of histological slides revealed the presence of *Mikrocytos* sp. (a few cells) in four oysters (two collected at Dayaowan Bay, two at Changdao) out of one hundred examined. These parasites, which had a nucleus of approximately 5 μm in diameter, were mostly present in the adductor muscle and digestive gland (Fig. 1); no parasites were found in other tissues. No infiltration of host hemocytes was observed. The normal architecture of tissues was not altered. In addition, no other parasites were observed.

3.2 PCR amplification and DNA sequencing

DNA from the four oysters out of one hundred that tested positive (4%) was amplified by PCR using the primer pair Mikrocytos-F+R. Four PCR amplification products were obtained after electrophoresis at 200 v for 30 min (Fig. 2). These 522 bp PCR products were sequenced, and BLAST analysis showed that they were 100% identical to the *Mikrocytos* sp. DFO-2002 18S rRNA gene sequence (GenBank accession number: DQ237912) and 89% identical to the SSU rRNA of *M. mackini* (GeneBank Accession Number: AF477623). We confirmed the identity of the product as *Mikrocytos* sp. based on the sequence information (Fig. 3). No PCR products were obtained using the MM-mack primer pair specific for *M. mackini*, and no amplification was obtained using DNA from oysters confirmed to contain *H. nelsoni* and *H. costale*.

3.3 In situ hybridization assays

ISH was used to confirm the presence of *Mikrocytos* sp. in the histology sections from *C. gigas* collected at Dayaowan Bay and Changdao. The MM-like probe reacted positively with the microcell parasite in the digestive gland tissue of the oysters (Fig. 4). The probe did not react with the negative control slide. The results of ISH showed a very weak positive signal, which was consistent with the result of histology (i.e., only a few cells were observed) (Fig. 1). The MM-like probe did not hybridize with *H. nelsoni* and *H. costale* in *C. gigas* tissue, whereas the MSX1347 and SSO 1318 probes did hybridize with *H. nelsoni* and *H. costale*, respectively (data not shown).

4 Discussion

The aim of this study was to examine oysters from the northern coast of the Yellow Sea for the presence of *Mikrocytos* sp. The parasite was detected in 4% (four out of one hundred) cultured Pacific oysters using histology, PCR and ISH. The oysters had a very low level of infection (less than five cells) based on the histological analysis. There was no typical focalised host response in the four infected oysters, which is similar to the result reported by Gagné et al. (2008). In this study, the parasite was mostly found in the digestive gland and very little in the adductor muscle. This suggests that *Mikrocytos* sp. may parasitize specific tissues in *C. gigas*. *Mikrocytos* sp. was not seen to alter the normal architecture of tissues or to cause mortality. Therefore, it is possible that the parasite did not significantly affect these oysters.

Results of the DNA-based diagnostic assays supported each other. The microcell parasite found in *C. gigas* hybridized with the *Mikrocytos*-like probe, which confirms that the parasite in *C. gigas* is *Mikrocytos* sp. No *M. mackini* were present in histological examination of tissue sections or in the PCR assay, although the probe can hybridize with *Mikrocytos*-sp.

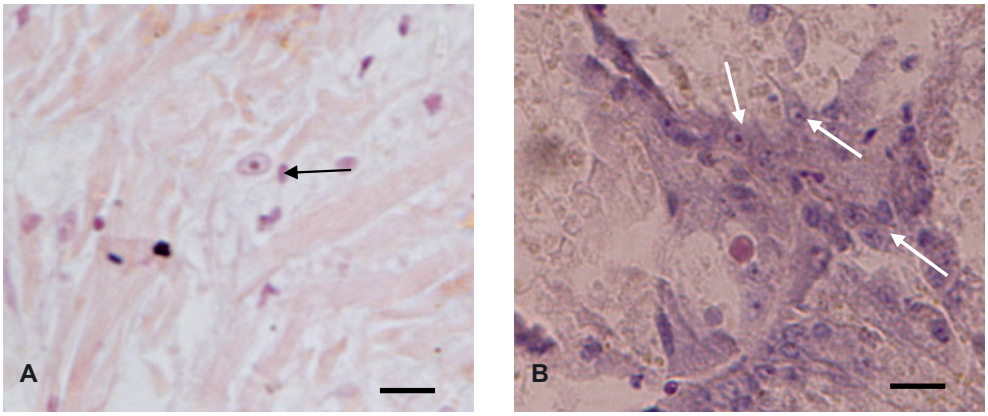

Fig. 1. *Crassostrea gigas* infected with *Mikrocytos* sp. parasites (arrows). Histological sections of *Mikrocytos* sp. with hematoxylin and eosin staining; A: adductor muscle, B: digestive gland of an oyster. Scale bar = 10 μm.

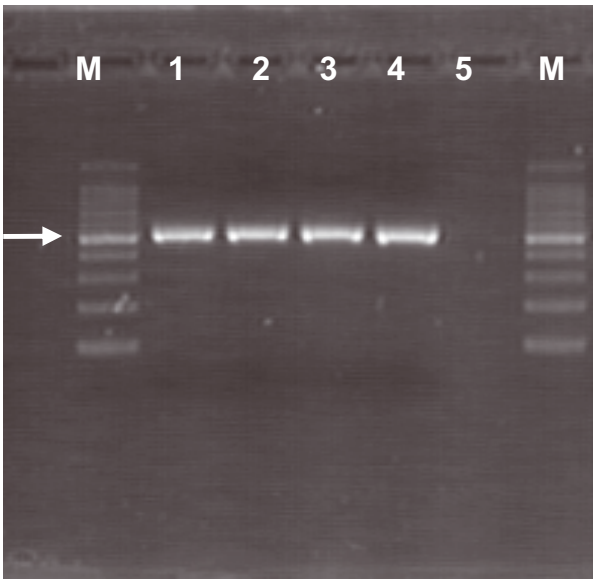

Fig. 2. Electrophoresis of PCR products using the primers Mikrocytos-F and Mikrocytos-R. Lane M: 100 bp ladder size markers; Lanes 1-4: PCR products from oysters collected at Dayaowan Bay (1, 2) and Changdao (3, 4); Lane 5: negative control (no DNA). Arrow indicates 500 bp.

and *M. mackini* (Gagné et al. 2008). The probe would need to be modified to a specific level if other parasites, such as *M. mackini*, are present in the same tissue section. In some cases, the intensity of infection appeared greater in the DNA analysis than that indicated by histology. This difference might be because of the very small size (about 5 μm in diameter) of the microcell parasite, the lack of a focalised host response (difficult to detect by histology), and the sensitivity of ISH. However, the few parasites detected and the difficulty in finding positive specimens with ISH suggests a low prevalence of *Mikrocytos* sp. along the coast of China.

It should be noted that the parasite was identified along the northern coasts of the Yellow Sea at latitudes between 37°N and 39°N, in January and May. The temperature conditions (2.5–15.5 °C) in this study are similar to those described for *M. mackini* infection, which usually occurs in the spring (Quayle 1982). Thus, it is possible that temperature is a controlling factor in the distribution of *Mikrocytos* sp. More studies are needed to examine the prevalence of the parasite in summer months in these areas.

This is the first report of the presence of microcells of *Mikrocytos* sp. in *C. gigas* along the coast of China. We do not completely understand why the parasite has only recently been detected in *C. gigas* in this area. However, we can propose several hypotheses: (1) In past years, a lack of shellfish health monitoring may have made it difficult to detect these parasites. Disease monitoring should be an essential part of any biosecurity program. This consists of regularly scheduled health evaluations of any part of hosts, environments, and pathogens. A wide range of techniques, including immunological, histopathological and molecular assays, should be applied to shellfish pathogen detection. Recently, pathogen detection methods for shellfish have been greatly developed in China. Accordingly, *Mikrocytos* sp. has only recently been found in China due to the development of diagnostic techniques for such pathogens, including PCR and ISH methods, which are more specific and sensitive to target organisms. (2) *Mikrocytos* sp. may have been transmitted from other marine areas into this area via unregulated domestic shellfish movements. For instance, the purchase of shellfish from a producer who sold infected stock could have introduced *Mikrocytos* sp. Once parasites are introduced into a certain area, they will probably proliferate rapidly and potentially threaten shellfish aquaculture. (3) *H. nelsoni* (MSX) could have been transported by ballast water between the Atlantic and Pacific oceans (Burreson and Ford 2004). *Mikrocytos* sp. was also probably introduced into China via infected tissue, ballast water, live oysters, or some other means of importation. In the future *Mikrocytos* sp. may exert greater pressure on aquaculture, thus the entire SSU rDNA sequence, ultrastructural observations, long-term investigations, prevalence studies, and a possible association between infection and mortality need to be studied further.

Acknowledgements. We thank Guize Liu et al. for collecting the oyster samples. This work was supported by the National Natural Science

Fig. 3. Nucleotide sequence of the parasite found in *Crassostrea gigas* compared with sequences of *Mikrocytos* sp. and *Mikrocytos mackini* from Genbank. Name 1-*Mikrocytos mackini* SSU rRNA gene; Name 2- *Mikrocytos* sp. 18S ribosomal RNA gene; Name 3- Sample. The Mikrocytos-specific primers used in this study are underlined.

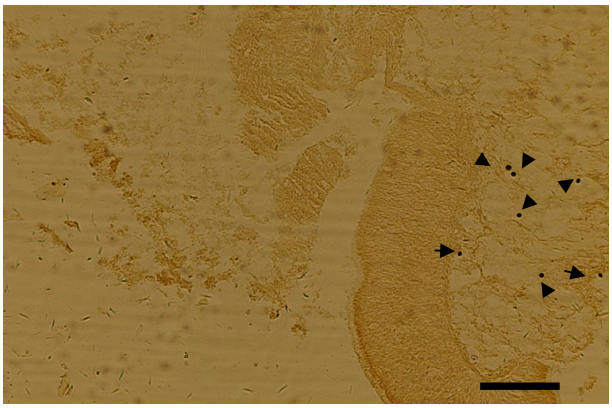

Fig. 4. In situ hybridization with the MM-like probe on the digestive gland of *Crassostrea gigas*. Scale bar = 100 μm.

Foundation of China under contract No. 30470275, National Special Grant of China under contract No. 908-01-ZH3.

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