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Cryptosporidium species from common edible bivalves in Manila Bay, Philippines

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ABSTRACT

Manila Bay is one of the major propagation sites of edible bivalves in the Philippines. Studies have shown that bivalves might be contaminated with human pathogens like the protozoan parasite *Cryptosporidium*, one of the major causes of gastroenteritis in the world. In this study, *Cryptosporidium* from four species of edible bivalves were isolated using a combination of sucrose flotation and immunomagnetic separation. Using direct fluorescent antibody test, *Cryptosporidium* oocysts were found in 67 out of 144 samples collected. DNA sequence analysis of the 18S rRNA gene of the isolates detected *C. parvum* and *C. hominis* (major causes of human cryptosporidiosis) and *C. meleagridis* (causes infection in avian species). Analysis of the 60 kDa glycoprotein gene further confirmed the genotypes of the *Cryptosporidium* isolates. This study is the first to provide baseline information on *Cryptosporidium* contamination of Manila Bay where bivalves are commonly cultured.
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1. Introduction

The protozoan parasite of the genus *Cryptosporidium* (phylum Apicomplexa) has emerged over the past decades as one of the major foodborne and waterborne pathogens worldwide. This parasite is among the major causative agents of gastroenteritis in humans and can potentially shorten the life span of immunocompromised individuals (Colford et al., 1996). *Cryptosporidium* in water and food items has been implicated in several human cryptosporidiosis outbreaks (Howe et al., 2002). In the Philippines, it has been found to affect cancer patients and has a significant prevalence among children (Rivera et al., 2005; Natividad et al., 2008).

The infective stage of *Cryptosporidium*, termed oocyst, can be disseminated successfully across several environmental matrices including water, food, and soil (Casemore, 1990). Environmental monitoring for *Cryptosporidium* oocysts in surface waters is made difficult by the low concentrations of organisms present due to dilution (Feng et al., 2003). Alternatively, filter-feeding invertebrates such as bivalves, which are known bioindicators or biomonitors, can concentrate *Cryptosporidium* oocysts present in the water (Gomez-Bautista et al., 2000). Hence, it is possible to recover higher number of oocysts from bivalves compared to those recoverable from surface waters.

Human consumption of bivalves is common in the Philippines. These bivalves serve as inexpensive source of protein for the Filipinos. In fact, some common edible bivalves are cultured in Manila Bay, which is known as one of the propagation sites of commercial fishes and other related sea foods in the metropolis. Bivalve production has been one of the sources of income in the coastal areas of Metro Manila. Because of this, more livelihood opportunities became available in these areas and therefore attracted more human settlements. This eventually resulted in increased human population as well as increased environmental contamination in these areas. Thus, humans may be at risk of infection due to consumption of bivalves that are contaminated with enteric pathogens like *Cryptosporidium*. In the Philippines, bivalves are preferred to be eaten lightly-cooked or even raw. However, studies have shown that *Cryptosporidium* oocysts may survive after cooking with steam or light fire (Hohweyer et al., 2013).

This study aimed to evaluate the occurrence of *Cryptosporidium* in common edible bivalves in the Philippines, which are harvested in the coastal waters of Manila Bay, and to obtain information on the species and genotype of *Cryptosporidium* contaminating these bivalves. This study is the first to provide baseline information on *Cryptosporidium* contamination of Manila Bay where bivalves are commonly cultured. This is also the first to report on genotyping of *Cryptosporidium* from bivalve samples obtained from culture sites in the Philippines.

2. Materials and methods

2.1. Bivalve sample collection

Non-probability purposive sampling was done in collecting a total of 144 samples of four commonly consumed marine bivalve species in the Philippines, *Perna viridis*, *Crassostrea iredalei*, *Venerupis philippinarum*

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and *Argopecten irradians*. These species were collected from bivalve propagation and culture sites around Manila Bay. The sample collection sites were Obando, Navotas and Malabon in the north part of Manila Bay and Parañaque, Las Piñas, and Cavite in the south part of Manila Bay. Strategic locations of the collection sites are shown in Fig. 1.

Each sample consisted of 15 live mussels that were selected at random for subsequent processing and analyses. For each batch, the bivalves were dissected and only the digestive glands were obtained. The mussel tissues were pooled (Molini et al., 2007) and homogenized in 5 ml distilled water, sieved through a double layer of gauze and pelleted by centrifugation (1000 × g, 4 °C, 10 min). The resulting sample homogenate was subjected to sucrose flotation to concentrate the *Cryptosporidium* oocysts.

2.2. Concentration of Cryptosporidium oocysts by sucrose flotation

Centrifugal sucrose flotation was performed similar to the method of the World Organization for Animal Health (2015). Each sample was transferred to 50 ml conical tube, followed by addition of 20 ml of 1 M sucrose solution. The tube was centrifuged at 1900 rpm for 15 min and the resulting interphase was aspirated and transferred to a new conical tube. Then, it was resuspended in 10 ml 1× phosphate buffered saline (PBS) prior to recentrifugation at 2300 rpm for 10 min. The resulting supernatant was removed, and 1 ml of 1× PBS was added to the pellet. This suspension was homogenized using a vortex mixer, transferred to a microfuge tube and stored at 4 °C until further use.

2.3. Immunomagnetic separation (IMS)

The resulting suspension from sucrose flotation was subjected to immunomagnetic separation (IMS) specific for *Cryptosporidium* species (Dynabeads®, Thermo Fisher Scientific Inc.) following the manufacturer's protocol.

2.4. Detection of Cryptosporidium oocysts

2.4.1. Modified Kinyoun acid-fast (AF) staining

Method on AF staining was adapted from procedures in WOAH (2015). Briefly, about 2–3 μl of the concentrated oocyst sample was smeared onto glass slide, air-dried and fixed with absolute methanol for 1 min. The slide was flooded with modified Kinyoun's carbol fuchsin and was stained for 5 min. It was rinsed briefly with 50% ethanol and rinsed thoroughly with distilled water. The slide was decolorized with 1% sulfuric acid for 2 min. It was rinsed again with distilled water and drained, counterstained with methylene blue for 1 min, rinsed with distilled water and then air-dried. The stained slide was examined using a light microscope (Olympus™, Germany). The presence of *Cryptosporidium* oocysts was confirmed by the presence of spherical bodies stained pink to red, with sizes ranging from 4 to 6 μm. Non-acid fast organisms or particulates were stained blue.

2.4.2. Direct fluorescent antibody (DFA) test

A commercial reagent, Invitrogen™ *Cryptosporidium* oocysts antibody FITC conjugate (Thermo Scientific, USA) was used according to the manufacturer's directions. Briefly, 3 μl of the concentrated oocyst sample was spread thinly onto a well of a multi-test slide and air-dried at room temperature. The sample was then fixed in absolute methanol for 5 min and air-dried again at room temperature. Five μl of the FITC-conjugated antibody solution was then added. After incubation at 37 °C for 30 min in the dark inside a humid chamber, the slide was washed with distilled water and air-dried. Mounting medium (glycerol:PBS at 60:40 v/v) was added before covering the slide with

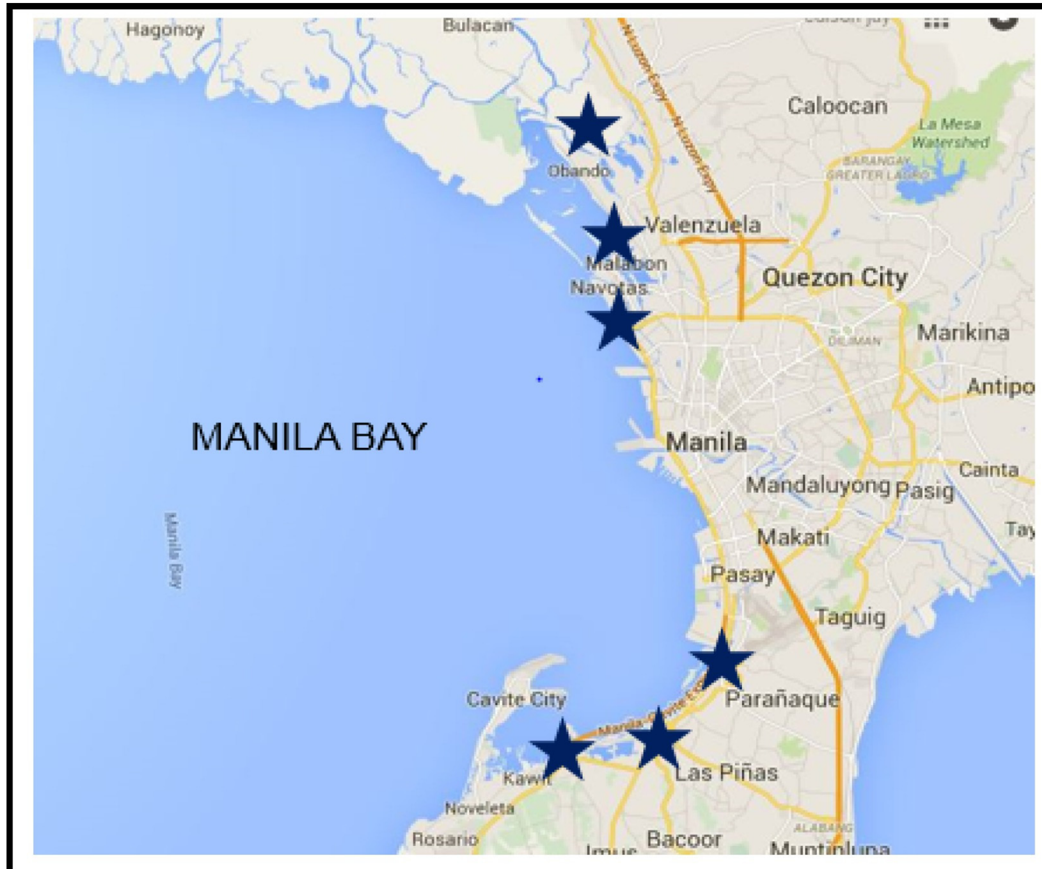

Fig. 1. Map showing the location of the sample collection sites in this study.

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