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First report of *Cryptosporidium hominis* in a freshwater sponge

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Abstract

Identification of *Cryptosporidium* oocyst is essential in ensuring water quality fit for human use, consumption, and recreation. This communication proposes the supplemental analysis of substrate-associated biofilms, in particular, freshwater sponges in improving case finding of waterborne-protozoan pathogens (WBPP) in environmental aquatic samples. In this study, a small portion of a mature freshwater sponge under the Genus *Radiospongilla* was subjected to microscopic and molecular analysis to identify the presence of *Cryptosporidium*. Microscopic screening with modified Kinyoun's staining (MK) and microscopic confirmation using direct antibody fluorescent testing (IFT) returned with *Cryptosporidium* spp. positive findings. Molecular investigation resulted in the confirmation of *Cryptosporidium hominis* upon sequencing of PCR products and phylogenetic analysis. This is the first report of a pathogenic protozoan, *C. hominis* isolated from a freshwater sponge. The results of this study provide evidence of the value of expanding water quality assessment strategies to the analysis of substrate-associated biofilms and sponges in improving case finding of WBPP in natural aquatic environments.

Keywords: Biofilms, Cryptosporidium, IFT, PCR, sponges, Protozoa

1. Introduction

Biofilms are widely-distributed in natural environments, grey-structures, and within biological systems like the oral cavity and digestive tract. This field of study has permeated a plethora of scientific

disciplines as it offers valuable information for understanding deeper the interactions of a wide spectrum of microbes in pure and multi-species community (Sivadon et al., 2019).

Sponges are the most ancient multicellular animals (basal Metazoa) in existence and filter water through its aquiferous system to acquire oxygen and nutrition from its environment. To date, sponge research includes biodiversity (Jakhalekar and Ghate, 2016), anatomic plasticity (Lavrov and Kosevich, 2016), microbial community interactions (He et al., 2014; Webster et al., 2004), antimicrobials (Laport et al., 2009; Tasdemir et al., 2007), inhibitory substances (Loaëc et al., 2017), and antiprotozoal compounds (Orhan et al., 2010).

Cryptosporidium remains to be the second leading cause of morbidity and mortality due to infantile diarrhea next to rotavirus worldwide (Parashar et al., 2003; Checkley et al., 2015) and ranks first in parasitic protozoan causing waterborne (Karanis et al., 2007; Baldursson and Karanis, 2011; Efstratiou et al., 2018) and foodborne outbreaks (Ahmed and Karanis, 2018; Ryan et al., 2018).

Efforts of the authors in the current study to establish the freshwater distribution of *Cryptosporidium* and other waterborne-protozoan pathogens (WBPP) in the Philippines by analyzing surface water (SW), sediments (BW), as well as substrate-associated biofilms (SAB), led to this novel finding. Upon survey of an aquaculture perimeter, a sponge was observed growing on the fishnet which house Nile tilapia *Oreochromis niloticus*. This sponge serves as food for the fingerlings and was collected as a substitute for SAB.

The aim of this study was to assess the sponge biomass for the potential presence of *Cryptosporidium* and elect freshwater sponges as potential biological reservoirs in the survey of pathogenic oocysts in natural aquatic environments.

2. Materials and methods

Lake Buhi (Fig. 1b) is located in the Bicol province of Southern Luzon, Philippines and is a major source of freshwater fish in the region where the world's smallest edible fish known as Sinarapan *Mistichtys luzonensis* is endemic. One mature sponge sample (Fig. 1c and d) labeled B1A5 SAB was harvested from a fishnet from coordinates 13°27'29.7"N 123°30'55.4"E, washed with sterile distilled water to remove non-adherent cells, a pinkie-sized portion of which was placed in a sterile conical tube with 50 mL sterile distilled water and processed within 24 hours. The 50 mL sponge suspension was vortexed for one minute to disrupt the biomass and liberate adherent cells and left to stand for five minutes to allow heavier debris to settle after which ten mL supernatant was aspirated, and centrifuged at 1500 x g for 15 minutes (US EPA, 2005). Eight mL supernatant was discarded while the remaining two mL with pellet was disturbed into a suspension, 25 µL of which was prepared into 1 X 1 cm diameter smears on sterile glass slides. Smears were screened for *Cryptosporidium* oocysts with Modified Kinyoun's Acid-Fast staining (MK), and additional microscopic confirmation was performed using Direct Antibody fluorescent testing (IFT) with Aqua-Glo™ G/C Direct Comprehensive Kit (Waterborne Inc. USA, 2010). DNA extraction of pellet suspension and *C. parvum* oocysts (P102C, Waterborne Inc., USA) was performed using Macherey Nagel Nucleospin™ following the manufacturer's protocol with slight modifications. Polymerase chain reaction was performed using 18s rRNA primers for *Cryptosporidium* following protocols from Pagoso and Rivera (2017) with slight modifications. Amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide and sent to commercial sequencing company (Macrogen South Korea). Sequences were aligned using Clustal W of Bioedit with a careful visual inspection of gaps and ambiguous sequences and was deposited in GenBank. Phylogenetic and molecular evolutionary analyses were conducted using Mega version 7 (Kumar et al., 2016) which was based on the best tree model with bootstrap set at 1000 replicates.

3. Results and discussions

Identification of freshwater sponge sample (Fig. 1c and d) was based on macroscopic morphology, qualifying the mature sponge sample under the genus *Radiospongilla* as being large sponges with finger-like surface projections, widely distributed in the Indian sub-continent, Sri Lanka, Indonesia, and the Philippines (Jakhalekar and Ghate, 2016). MK staining was positive for *Cryptosporidium* spp. oocysts (Fig. 2a and b), which fits morphologic descriptions and size of 4-6 µm (CDC DPDx Cryptosporidiosis). Further microscopic confirmation using IFT also returned positive results where oocysts exhibited apple-green fluorescence, with oocysts size and morphology representative of *Cryptosporidium* (Fig. 2c and d). The relatively easy visualization of oocysts in this low volume of sample through MK and IFT staining are clear indications of high contamination of the sponge sample with *Cryptosporidium* oocysts.

PCR results showed banding in the 350 bp region when compared to a 1 kb ladder with the band being aligned with *C. parvum* positive control (Fig. 2e). Phylogenetic analysis suggested through maximum likelihood that isolate B1A5 SAB is *C. hominis* and was provided with GenBank accession number MK989995 (Figure 3).

Environmental distribution of *Cryptosporidium* spp. have been reported in watersheds (Masangkay et al., 2016), sediments (Kong et al., 2017), and in tap water (Watanabe et al., 2005). In humans, *C. parvum* and *C. hominis* have been linked to outbreaks (Horne et al., 2017) and were recently reported in freshwater bivalves for human consumption in the Philippines (Pagoso and Rivera, 2017). *C. viatorum* and *C. meleagridis* although less commonly isolated, have been detected in immunocompromised individuals (Wesolowska, et al., 2016; Adamu et al., 2014).

The biofilm matrix has been reported to associate a variety of unicellular species (Dopheide et al., 2009; Puzon et al., 2009; Chang et al., 2010; Valster et al., 2009; Wey et al., 2012). To date, although in vitro culture of *Pseudomonas aeruginosa* in an aquatic biofilm system has been demonstrated to facilitate the

progression of *C. parvum* oocyst to several developmental stages (Koh et al., 2013), there is no existing report on the natural-association of *Cryptosporidium* to environmental biofilms. Similarly, sponges can act as biological indicators of water pollution (Kallok, 2017), but just the same, no evidence for the natural-association of *Cryptosporidium* has been presented.

Temporal accumulation of *Cryptosporidium* oocysts in a mature sponge can be facilitated by the filtration of large volumes of water through its aquiferous system (Lavrov and Kosevich, 2016). In addition, calcium, which is essential for the germination of sponges (Ostrom and Simpson, 1978) can facilitate enhanced interactions between particles (Luo et al., 2016) and surfaces and the outer wall of *Cryptosporidium* oocysts through divalent cation bridging mechanism (Sarkhosh et al., 2019). The Lake Buhi results returned 20% (1/5) MK and 20% (1/5) IFT positivity from 50 mL SW and BW samples, respectively (Masangkay, unpublished data). These observations complement the *Cryptosporidium* positive microscopy results from our mature sponge sample. Further, PCR banding in the 350 bp region of both B1A5 SAB and *C. parvum* DNA isolates provided molecular confirmation of the isolation of *Cryptosporidium* spp. (Pagoso and Rivera, 2017), which upon sequencing and phylogenetic analysis (Fig. 3) was found to be *C. hominis* and was allocated with GenBank accession number MK989995.

Sponges have been elected in the analysis of genetic material through trapped DNA from the surrounding environment (Pennisi, 2019), this is valuable in that even in cases of degenerated oocysts; sponges may still be able to provide evidence of *Cryptosporidium* association through molecular testing. These sponge-associated oocysts are provided with UV light protection by the sponge biomass whereby viability can be maintained for longer periods thereby increasing the potential for both human and zoonotic infections (DiCesare et al., 2012) and is a potential hazard to Lake Buhi's residents, fishermen, and tourists. Results of the study show that freshwater sponges could be utilized as bioindicators of the microbiological quality of natural water resources. The findings of this research provide evidence-based

rationale to expand water quality assessment strategies to testing SAB and sponges whenever applicable in addition to SW and BW analysis in improving WBPP detection.

4. Conclusions

- This short communication provides the first report of the presence of *Cryptosporidium hominis* in a freshwater sponge.
- Biofilms, and in this case, a mature sponge has the potential to act as a biological reservoir for harmful protozoan species with outbreak potential.
- Expanding water quality assessment strategies to biofilms and sponges may improve the determination of water resource quality and facilitate the formulation of the necessary interventions in the prevention of the spread of waterborne protozoan pathogens.

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Competing interests

The authors declare no competing interests

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Figure

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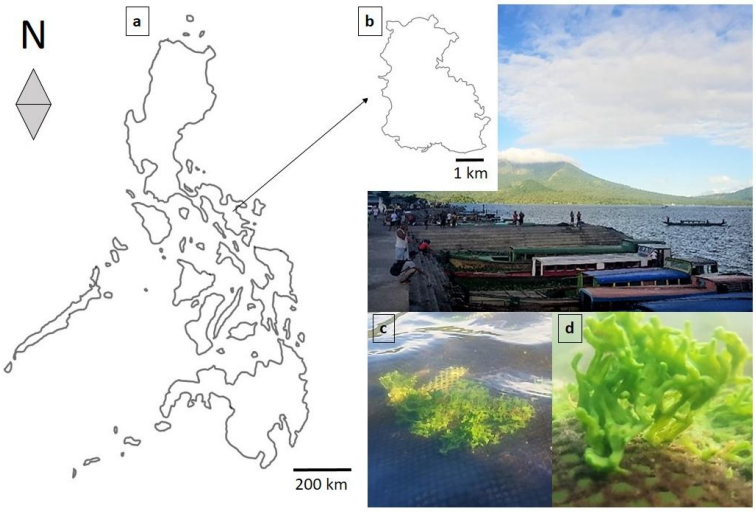

Fig. 1. Geographic representation and pictures of sampling site and sample matrix. **a** Map of the Philippines, **b** Lake Buhi, **c** mature sponge growing on a fishnet, **d** submerged shot of collected sponge sample with distinct finger-like surface projections.

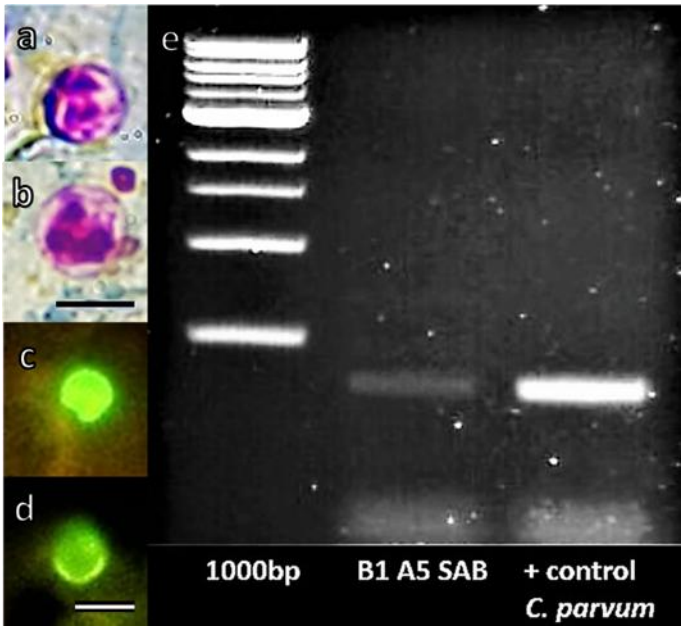

Fig. 2. *Cryptosporidium hominis* oocysts from a mature sponge. **a** to **d** different individual oocysts from MK and IFT stained smears, **a** and **b** MK positive 1000X magnification, **c** and **d** IFT positive 400X magnification, Scale bars at 5 µm, **e** PCR positive result of B1A5 SAB and *C. parvum* positive control with 1kb ladder

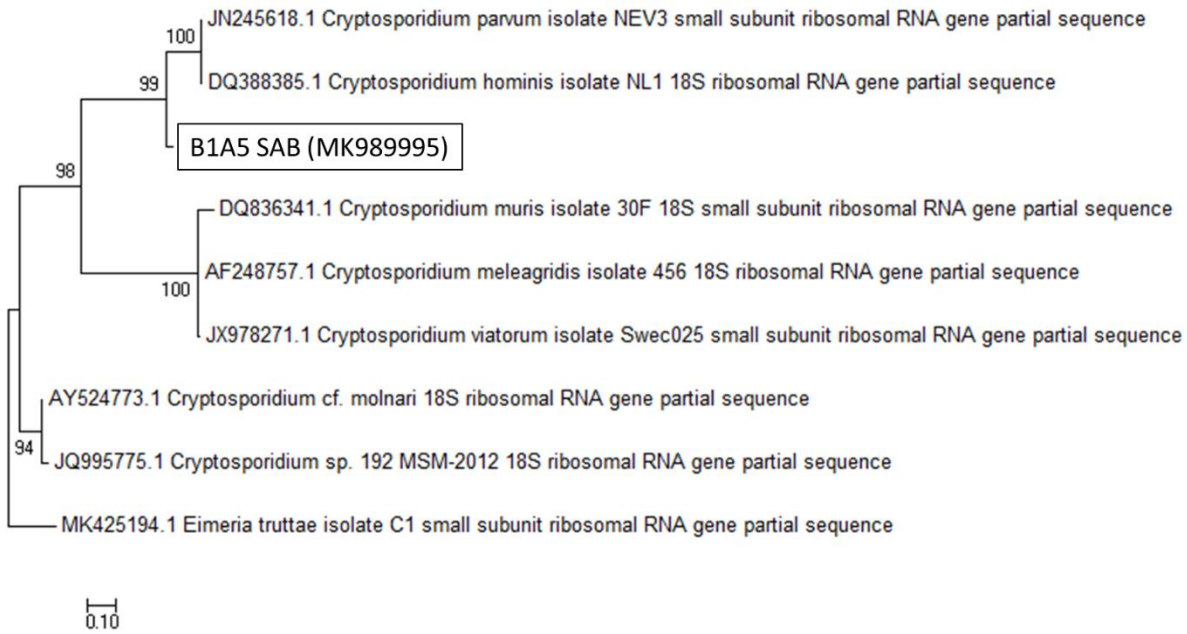

Fig. 3. The phylogenetic tree shows possible relationships with reference sequences of *Cryptosporidium* spp. to B1A5 SAB isolate (GenBank accession number MK989995) from the freshwater sponge in the fishnet of Lake Buhi, Bicol, Philippines. Bootstrap showing 1000 replicates. The tree was rooted using *Eimeria truttae* as outgroup.