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1 **First report of *Cryptosporidium hominis* in a freshwater sponge**

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26 **Abstract**

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

40

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

42

43 **1. Introduction**

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

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

48 Sponges are the most ancient multicellular animals (basal Metazoa) in existence and filter water through  
49 its aquiferous system to acquire oxygen and nutrition from its environment. To date, sponge research  
50 includes biodiversity (Jakhalekar and Ghate, 2016), anatomic plasticity (Lavrov and Kosevich, 2016),  
51 microbial community interactions (He et al., 2014; Webster et al., 2004), antimicrobials (Laport et al.,  
52 2009; Tasdemir et al., 2007), inhibitory substances (Loaëc et al., 2017), and antiprotozoal compounds  
53 (Orhan et al., 2010).

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

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

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

67

## 68 2. Materials and methods

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

91

### 92 3. Results and discussions

93 Identification of freshwater sponge sample (Fig. 1c and d) was based on macroscopic morphology,  
94 qualifying the mature sponge sample under the genus *Radiospongilla* as being large sponges with finger-  
95 like surface projections, widely distributed in the Indian sub-continent, Sri Lanka, Indonesia, and the  
96 Philippines (Jakhalekar and Ghate, 2016). MK staining was positive for *Cryptosporidium* spp. oocysts (Fig.  
97 2a and b), which fits morphologic descriptions and size of 4-6  $\mu\text{m}$  (CDC DPDx Cryptosporidiosis). Further  
98 microscopic confirmation using IFT also returned positive results where oocysts exhibited apple-green  
99 fluorescence, with oocysts size and morphology representative of *Cryptosporidium* (Fig. 2c and d). The  
100 relatively easy visualization of oocysts in this low volume of sample through MK and IFT staining are  
101 clear indications of high contamination of the sponge sample with *Cryptosporidium* oocysts.

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

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

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

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

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

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

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

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141

#### 142 **4. Conclusions**

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

150

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157

#### 158 **Competing interests**

159           The authors declare no competing interests

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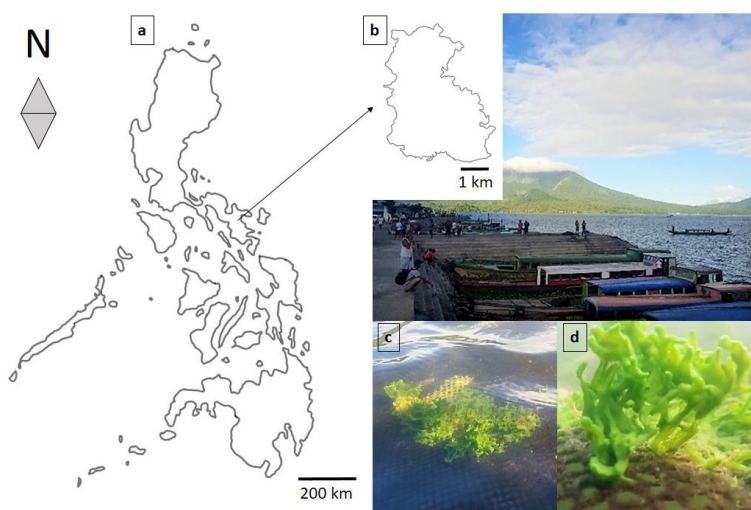
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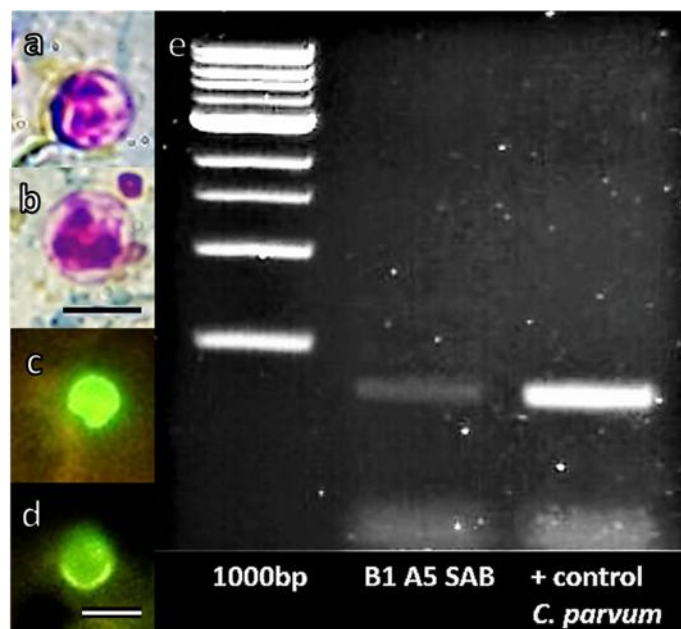


# Figure

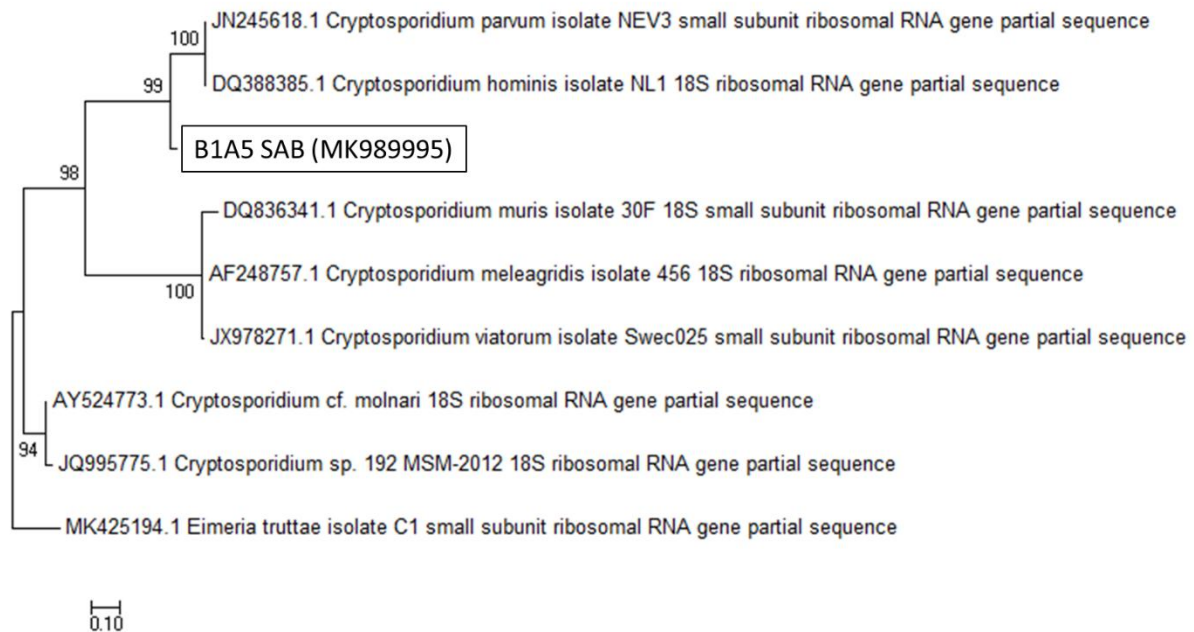
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**Fig. 1.** Geographic representation and pictures of sampling site and sample matrix. **a** Map of the Philippines, **b** Lake Buhi, **c** mature sponge growing on a fishnet, **d** submerged shot of collected sponge sample with distinct finger-like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