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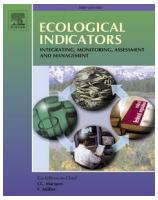

Original Articles

Molecular investigation of waterborne protozoan contamination using marine *Demospongiae*

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ABSTRACT

Sponges play important role within aquatic ecosystems due to their diverse abilities including filter-based feeding mechanisms. Hence, this study evaluated the potential use of sponges as ecological biomonitors for water safety surveillance, especially in the presence of Waterborne protozoan pathogens WBPP. Sponge specimens were collected from different Qatari marine ecosystems and subjected to gDNA extraction and real-time PCR using specific primer sets for the most common WBPP. Two sponges from the coastal marine ecosystems were found to be positive for *Blastocystis* sp., and one sponge was positive for *Dientamoeba fragilis* within offshore site. No *Cryptosporidium* spp., *Giardia duodenalis*, nor *Toxoplasma gondii* were detected. Further genotyping analysis revealed that the *Blastocystis* sp. positive samples were subtype ST3 (allele 34), which matched local clinical isolates and *D. fragilis* specimen was unambiguously clustering with Genotype 2. In conclusion, this study demonstrates the role of marine sponges as ecological biomonitors for WBPP screening and provide insights into these pathogens widespread and their potential transmission to marine and terrestrial organisms including human.

1. Introduction

The phylum Porifera (designating "pore-bearing" entity) is considered to enclose one of the oldest poly-celled organisms with a fossil record dating back to the Precambrian times (Abdelmohsen et al., 2014). It includes around 150 sponges living in freshwater with the rest found within the sea/ocean or brackish water. They are subdivided into four major classes of sponges: Hexactinellida, Homoscleromorpha, Calcarea, and Demospongiae (Lukowiak et al., 2022). The latter comprises 85% of the world's sponges with broad morphological plasticity in size, coloration (red, orange, blue, yellow, purple, etc.), shapes, and location (found at all depths) (Esposito et al., 2022). Consequently, with its widespread occurrence and exposure to different aquatic ecosystems, Demospongiae have provided significant services in the evolutionary history of marine ecosystems. Indeed, they are one of the first evolving forms of multicellular life (Pennisi, 2019), producing different sources for bioactives (Giraldes et al., 2020) and components for industrial materials processing (Görlich et al., 2020), as well as playing the role of functional bio-indicators of the environmental health (Moitinho-Silva et al., 2017). Demosponges are filter-feeding animals with an active filter-based mechanism that circulates large volumes of water through their aquiferous system to obtain the needed nutrition (Steffen et al., 2022). They are considered hence, living sieves with their dynamic pumping and are more exposed to environmental threats than most other animals. In other words, these filter-feeding animals can reflect the distribution of particles in the water surrounding them (Gross, 2021) and act as indiscriminate traps for the adjacent microorganisms that may pose a public health concern, like the waterborne-protozoan pathogens (WBPP).

The WBPP have been implicated in numerous waterborne disease outbreaks worldwide, with Cryptosporidium spp. being responsible for 192 outbreaks and Giardia *duodenalis* for 48 outbreaks. These protozoa are known to be the primary fecal parasitic pollutants in aquatic ecosystems (Karanis et al., 2006). In addition, water outbreaks associated with Dientamoeba fragilis have also been reported in Europe and in Oceania, as well as Toxoplasma gondii and Blastocystis sp. within the

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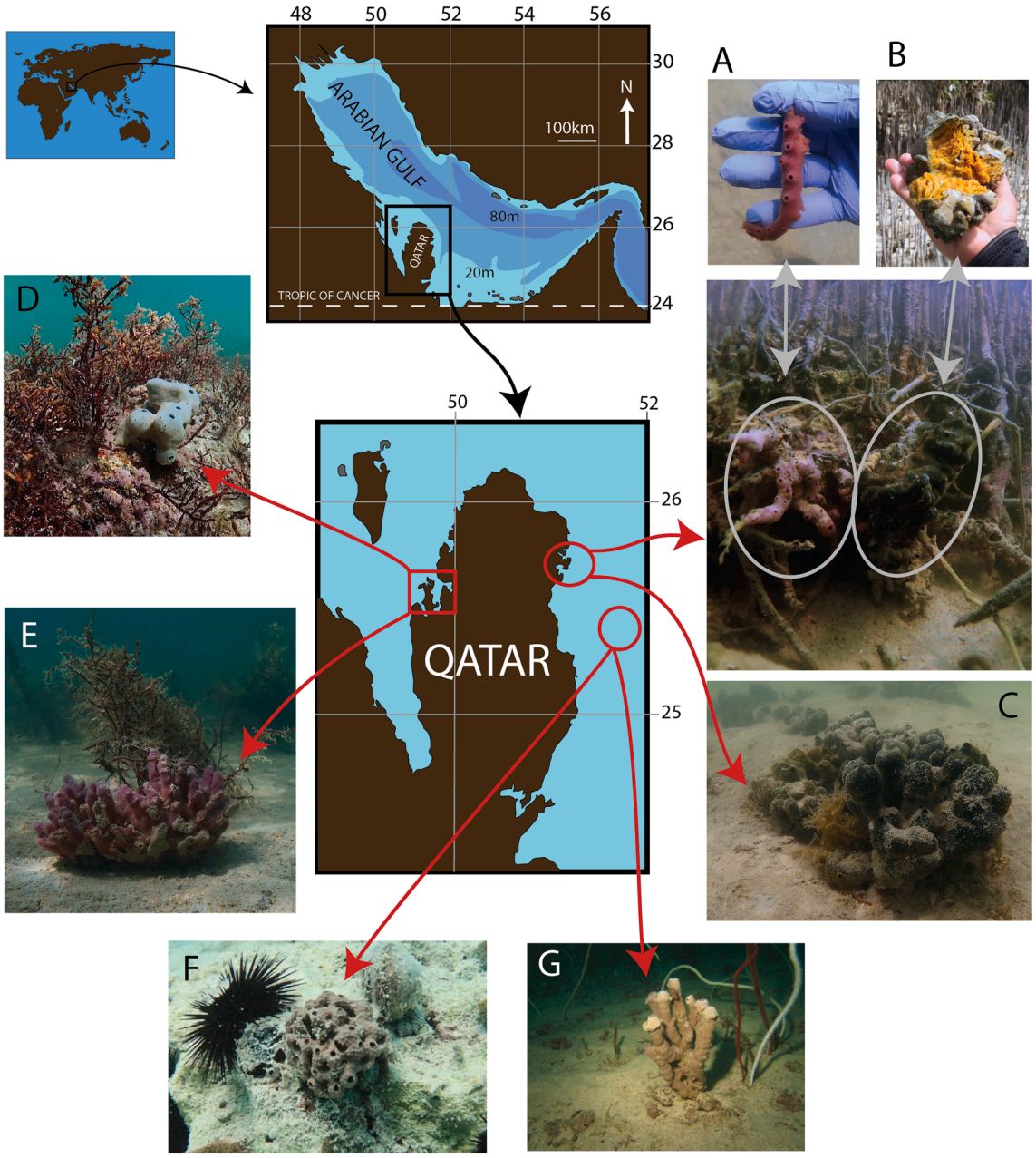

Fig. 1. Representative map of the different sampling sites. A/B/C are sponge specimens from the hyperarid mangrove ecosystem; D/E are sponge specimens from hypersaline shallow subtidal ecosystem; F/G are sponge specimens from offshore/oyster bed ecosystem.

Americas (Ma et al., 2022). The pathogens can be entrapped in different parts of the sponge structure by crossing its aquiferous canals, lodging within the matrix, or settling on its external walls (Masangkay et al., 2022). The accumulation of the protozoan (oo) cysts on and within the different sponge matrix could therefore maintain the protozoa occurrence, additionally the UV light protection that the sponge biomass provide would enhance consequently the spread the infecting parasite forms zoonotically to the marine organisms and indirectly to the terrestrial animals. The (oo) cysts of the mentioned WBPP have already demonstrated extended viability in different water resources, thus, contributing to their efficient spread (Betancourt and Rose, 2004). Humans, especially, are prone to WBPP infections through multiple transmission routes (e.g., zoonotic, foodborne, waterborne) and may adverse very severe clinical conditions if exposed to contaminated water sources (Sjöström et al., 2022). Most of the reported WBPP lead to severe intestinal manifestations (Boughattas et al., 2017a), except *T. gondii,* which is mainly involved in congenital and ocular manifestations (Boughattas et al., 2011; Dubey et al., 2021).

Microbiologically contaminated seawater has been found in a neighboring Middle East country with evidence of sanitation-related infections (Hilles et al., 2014). Hence, the need to scrutinize and monitor water sources for WBPP contamination to avert any outbreak scenarios. In Qatar, previous studies reported a high prevalence of WBPP in different local populations: new and settled Immigrants (Abu-Madi et al., 2015), pediatrics admitted to emergency units (Boughattas et al., 2017a), and even stray animals (Boughattas et al., 2017b). However, research gaps about their transmission are still to be fully understood. The recent identification of Cryptosporidium and Giardia spp. in freshwater sponges (Masangkay et al., 2020; 2022), raises red flags for public health concerns. Indeed, WBPP can lead potentially to morbidity cases and/or even unfortunate mortality records within the general public particularly the immunoexpressed subjects,

Table 1
Details of the different used primer sets for protozoa detection.

Parasite	Primers/Probe	Sequence 5′-3′	Reference
Blastocystis sp.	FwdS1	GGTCCGGTGAACACTTTGGATTT	Abu-Madi et al., 2015
	RvsS2	CCTACGGAAACCTTGTTACGACTTCA	
Cryptosporidium spp.	SCL2	CAGTTATAGTTTACTTGATAATC	Boughattas et al., 2017a
	SCR2	CAATACCCTACCGTCTAAAG	
	CrySB	FAM/CCGTGGTAATTCTAGAGCTA/BHQ	
Dientamoeba fragilis	DF3	GTTGAATACGTCCCTGCCCTTT	Stark et al., 2006
	DF4	TGATCCAATGATTTCACCGAGTCA	
	Probe	FAM-CACACCGCCCGTCGCTCCTA	
Giardia duodenalis	Gd-80F	GACGGCTCAGGACAACGGTT	Verweij et al., 2004
	Gd-127R	TTGCCAGCGGTGTCCG	
	105T	FAM-CCCGCGGCG/ZEN/GTCCCTGCTAG	
Toxoplasma gondii	Frwd	GCATTGCCCGTCCAAACT	Wahab et al., 2010
	Rvs	AGACTGTACGGAATGGAGACGAA	
	Probe	FAM-CAACAACTGCTCTAGCG-BHQ1	

Consequently, the current study aims to investigate the temporal accumulation of major WBPP in marine sponges by detecting the trapped forms of *Blastocystis* sp., *Cryptosporidium* spp., *D. fragilis,* Giardia sp., and *T. gondii,* with the ultimate goal of assessing the extent of water pollution. Molecular genotyping analysis of the potential parasites may shed light on their epidemiological transmission and potential health risk. The findings will help evaluate the potential use of sponges as ecological biomonitors for water safety surveillance, particularly when WBPP contamination is present within environmental resources.

2. Material and methods

2.1. Sampling

Specimens of Demospongiae (n = 20) were collected by snorkeling and freediving from different locations of Qatari marine ecosystems (hyperarid mangrove ecosystems, hypersaline shallow subtidal ecosystem and offshore/oyster beds ecosystem) by the experts of the Environmental Sciences Center (ESC) at Qatar University as reported within the generated illustrator map (Fig. 1). Field exploration did not target any endangered or protected species; hence specific permissions are not applicable for Porifera specimen sampling. The collected sponges were recoded, photographed and then were stored in Sea Water within the Qatar University (QU) biorepository until downstream analysis. Their identification was based on upper taxonomic levels using the Porifera systematics (Hooper and Van Soest, 2002; Giraldes et al., 2020).

2.2. DNA extraction

From each sponge specimen, pieces of ca. 1 cm^3 were cut and rinsed three times by PBS buffer for 5 min at 4000×*g* before their homogenization using the TissueRuptor II (Qiagen) at low speed for 10 sec. The lysates were then subjected to genomic DNA extraction as described elsewhere (Boughattas et al., 2021) using modified protocol of Genomic Tips kit (Qiagen). The quality of the extracted DNA was checked using nanodrop ratio A260/A280 and agarose gel electrophoresis.

2.3. Molecular detection

The different parasites were screened by Real-Time PCR using TaqMan chemistry for Cryptosporidium spp., D. fragilis, *G.* duodenalis, and T. gondii (Boughattas et al., 2017a; Wahab et al., 2010) and SyberGreen chemistry for Blastocytis sp. (Abu-Madi et al., 2015) (Table 1). To avoid contamination and cross-over reactions, sample processing; extraction; amplification; purification, and sequencing preparations were carried out on a physically separate laboratory benches. Amplification reactions were carried out by the AriaMx Real-time PCR System (Agilent©).

2.4. Sequencing and phylogenetic analysis

The detected parasites were subjected to genotyping analysis by direct bi-directional sequencing of the 18S rRNA gene (Scicluna et al., 2006; Cacciò et al., 2016) at Macrogen© (South Korea). Analysis, cleaning, and editing of the sequences were achieved by BioEdit software. Multiple alignments with homologous sequences were achieved by the MAFFT software, followed by phylogenetic analysis using the Maximum-Likelihood ML method by the MEGA X software with 1000 bootstraps. The generated nucleotide sequences within the current work have been deposited into the GenBank database under accession numbers: OQ729719, OQ729720.

3. Results

From the total 20 specimens collected, four sponges were unclassified Demospongiae, four were Suberidae, three were Chalinidae, two were Tethyidae, two were Darwinellidae, and one from each of the following classes: Callyspongidea, Clionadae, Dysideidae, Petrosiidae, and Tedaniidea. The molecular screening of the different protozoa by qPCR didn't reveal the presence of *Cryptosporidium* spp., *G. duodenalis,* nor *T. gondii* in any of the different sponges' specimens. Withal, amplification curves provided evidence of the presence of *Blastocystis* sp. in 2 of the 20 samples (one Suberidae (*Suberites* sp.) from the mangrove ecosystem and one Chalinidae (*Haliclona* sp.) from the shallow subtidal ecosystem), as well as the presence of *D. fragilis* contaminating one Demospongiae (*Demospongea* sp.) specimen within the offshore site. The three infested sponges were collected at different timings and from three different geographic zones (Table 2).

The *Blastocystis* sp. positive samples were then subjected to DNA barcoding subtyping using RD5 and BhRDr set of primers. The targeted region of 600 bp was successfully amplified for both samples and unambiguously sequenced. When comparing both specimens' sequences over the trimmed 572 bp region, only one SNP was observed substituting

Table 2
Details of the contaminated specimens.

Specimen code	Sponge Family	Date of collection	Zone of collection	Detected parasite	Identified genotype
1164	Demospongiae	10.02.2018	Offshore	*Dientamoeba fragilis*	Genotype 2
1432	Suberidae	12.03.2017	Mangrove	*Blastocystis* sp.	Subtype ST3
1583	Chalinidae	17.12.2018	Shallow subtidal	*Blastocystis* sp.	Subtype ST3

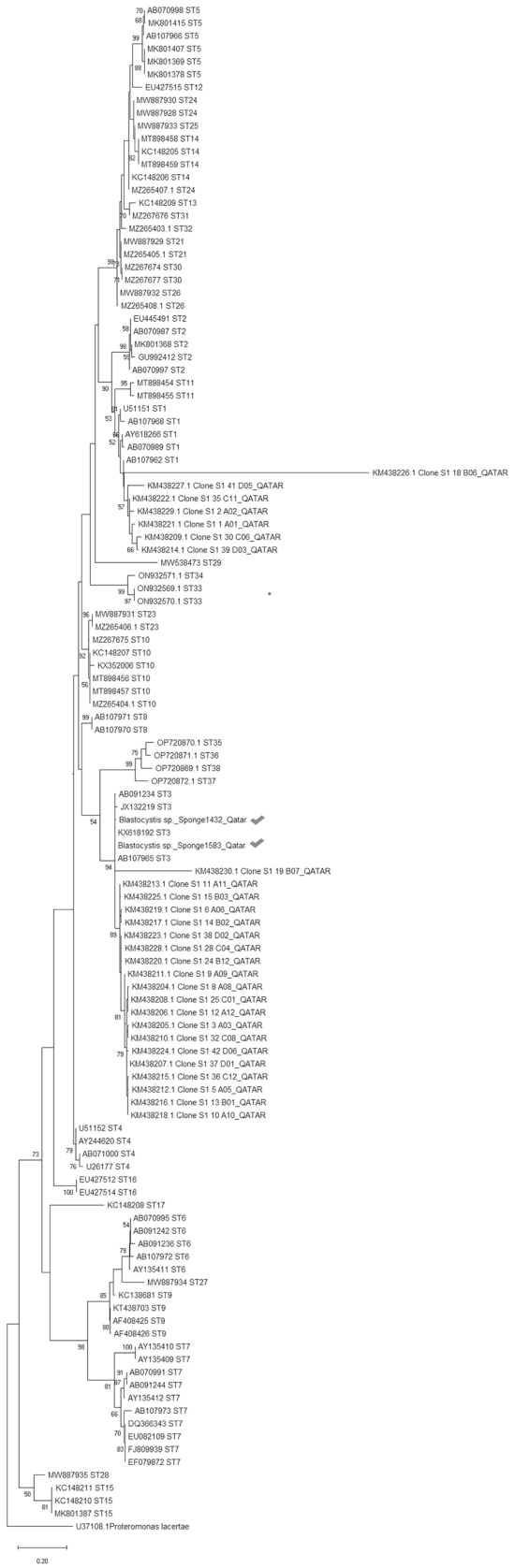

Fig. 2. *Blastocystis* sp. reference phylogenetic tree. Maximum likelihood phylogenetic tree (model HKY + G) inferred from the different reference *Blastocystis* subtypes of the SSU rRNA gene and the Sponge isolates. The tree is artificially rooted in *Proteromononas lacerate* sequence. Numerical values indicate bootstrap support. Only values above 50% are depicted.

the T in specimen 1583 by a C at the position of the 299th nucleotide of specimen 1432. When assessed with homologous sequences previously deposited in the NCBI database, the generated *Blastocystis* sp. sequences exhibited high similarity: 100% for specimen 1583 (Accession number OQ729720) and 99.83% for specimen 1432 (Accession number OQ729719) with the ST3 subtype. The allele identification with the molecular typing and microbial genome diversity database (PubMLST) revealed an exact match found with Subtype ST3, Allele 34. Furthermore, within the phylogenetic analysis, both sponges' sequences were aligned with the different reference subtype sequences as well as with previously deposited sequences from the same country. Our specimens cluster again with Reference subtype ST3 sequences (Accession numbers AB107965 and KX618192) as well as with previously identified ST3 within local clinical isolates (Fig. 2).

The *D. fragilis* specimen was also subtyped by targeting its 18S rRNA gene using DF322F and DF687Rev primers. The gene was successfully amplified, and a 366-bp band size was observed on agarose electrophoresis. The generated sequence was then compared to previously deposited *D. fragilis* isolates in the NCBI database, and high similarity was observed, with 100% of Percent identity determined using the BLAST-N approach. The sponge specimen sequence was aligned with several *D. fragilis* isolates from different hosts (human and animals) and from different geographic locations, including the two reference sequences for each of the reported *D. fragilis* genotypes: Genotype 1 (AY730405.1) and Genotype 2 (U37461.1). The conducted ML phylogenetic analysis strongly supported (86%) the clustering of our marine sponge isolate with Genotype 2 isolates and its unambiguously distance from Genotype 1 isolates (Fig. 3).

4. Discussion

This study presents the first report of waterborne-protozoan pathogens in marine Porifera species with the identification of *Blastocystis* sp. and *D. fragilis* accumulation within Demospongiae specimens from coastal and offshore sites of Qatar. Scare studies on parasites within sponges are available with primarily observational and culture-dependent approaches reporting previous *Amoebozoa* contamination from Red Sea as well as *Neoparamoeba aestuarina* spoliation from Brazilian Coasts (Rinkevich et al., 1998; Custodio et al., 1995). However, with the development of new approaches, the opportunistic protozoa *Giardia* and *Cryptosporidium* were recently identified from freshwater sponges "*Spongilla*" within Lake Buhi in Philippines (Masangkay et al., 2020; 2022). According to the authors, the observed contamination of their lithosphere surface with human an animal feces pathogen as well as their region extreme weather magnify the pattern of ecosystems pollution. Since the reported abundance of WBPP within the State of Qatar (Boughattas et al., 2017), we targeted the investigation of the same described circulating species including *Blastocystis* sp*, Cryptosporidium* sp., *D. fragilis, G. duodenalis,* and *T. gondii*. The current work reports only *Blastocystis* sp. and *D. fragilis* accumulation within the endemic *Demospongiae* inhabiting the Qatari ecosystems.

Blastocystis sp. is believed to be the most widespread non-fungal microeukaryote present in hosts gastrointestinal tract (Abe, 2004) with major fecal-oral spread route as well as zoonotic, foodborne and waterborne routes (Rauff-Adedotun et al., 2021). Indeed, the stramenopile was identified from different water sources worldwide (Attah et al., 2023), suggesting their humans and/or animals' fecal contamination with even drinking waterborne outbreak occurrence (Maçin et al., 2017). Moreover, the viability of the *Blastocystis* sp. forms has been reported in water with a wide temperatures range (Ahmed and Karanis, 2018), as well as their resistance to the conventional chlorine and hydrogen peroxide treatments (Martín-Escolano et al., 2023). Within the State of Qatar, the protozoan has been identified in 71.1% of the workers with a predominance of subtype ST3 (Abu-Madi et al., 2015), which is believed to be a pathogenic strain linked to higher inflammation rates (Fréalle et al., 2015). When analysing the global

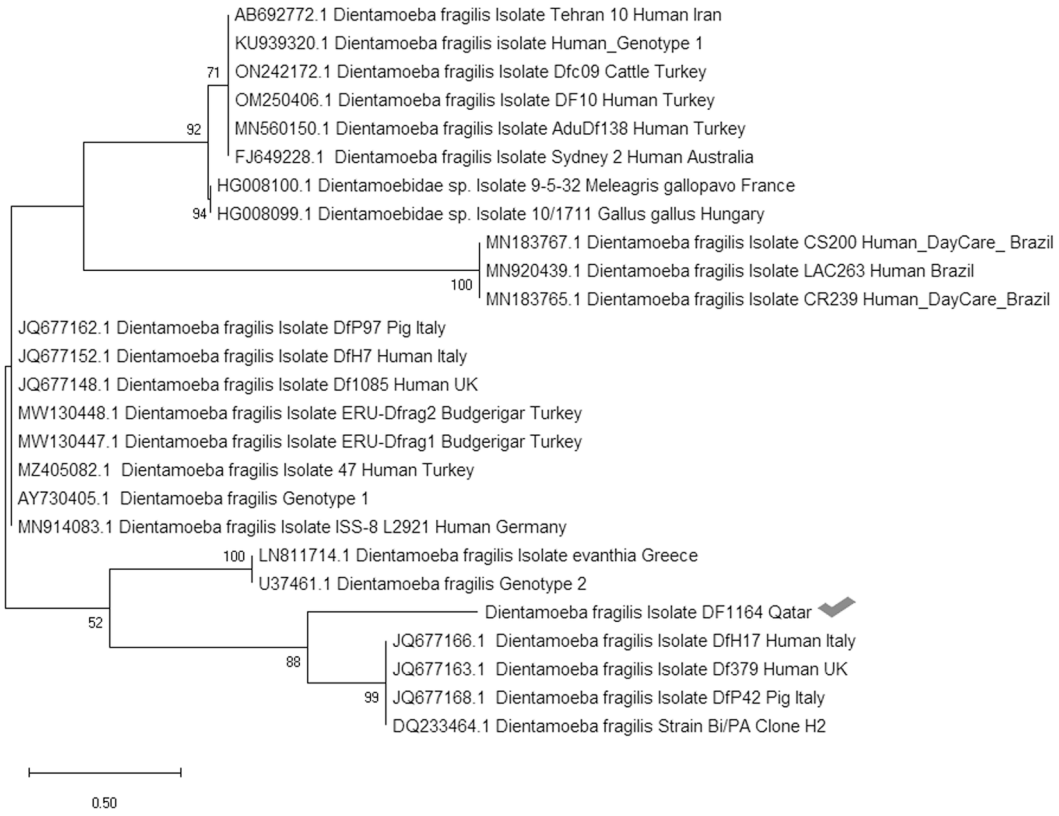

Fig. 3. *Dientamoeba fragilis* unrooted Maximum likelihood phylogenetic tree (model K2). It represents different isolates' genotypes of the SSU rRNA gene and the Qatari marine sponge isolate. Numerical values indicate bootstrap support, and only values above 50% are depicted.

variations of ST3, the allele 34 detected within our sponge specimens is reported to be the most prevalent allele in Asia (Nemati et al., 2021), Europe (Hernández-Castro et al., 2023), Africa (Ahmed et al., 2022), and America (Jiménez et al., 2022). The identification of human-prevalent and pathogenic subtype within the current work highlights the probability of waterborne transmission of this parasite within the region.

D. fragilis is second to *Blastocystis* sp. as a worldwide agent colonizing the hosts digestive tract and its infection involves a wide spectrum of gastrointestinal impairments (van Gestel et al., 2019). Yet the spread mechanism of the protozoon is still not fully understood with the suggestion of fecal-oral transmission route (Cacciò, 2018). The parasite was believed initially to be unable to survive outside the host's body and in the environment, making it difficult to identify though coproscopic identification (Abu-Madi et al., 2017). The use of the molecular tools has enabled a better understanding of the epidemiology of *D. fragilis* by identifying it in various non-human hosts, such as Gorilla (Stark et al., 2008), brown/Norway rat (Galán-Puchades et al., 2021), pigs (Crotti et al., 2007; Cacciò et al., 2012), cats and dogs (Chan et al., 2016), rabbit, horse, sheep, goat (Jirku et al., 2022), cattle (Yildiz and Erdem, 2022), and pet budgerigars birds (Yetismis et al., 2022). Moreover, *D. fragilis* was detected in commercially packed ready-to-eat salads in Italy (Caradonna et al., 2017), untreated water bodies (Stark et al., 2012) as well as treated water sources (Berglund et al., 2017), and even within drinking waterborne outbreaks in Turkey, New Zealand, and Finland (Ma et al., 2022), which strongly suggest its zoonotic importance. It is still unknown if the pathogenicity and the spread of the parasite is correlated to its genetic diversity represented so far by two genotypes (Cacciò et al., 2016). The variant identified within our sponge specimen belongs the rarest identified group, Genotype 2. No previous data about the genotype distribution of this parasite within local populations is available, so correlations cannot be emitted yet.

The results of this study support that Demospongiae species are efficient traps for pathogens within marine ecosystems. Marine sponges are known as symbiotic to a very diverse and complex microbial communities that may constitute up to 40–60% of the total sponge biomass (Najafi et al., 2018). An evolutive symbiosis with great importance for sponges because of their involvement in vitamin synthesis, ultraviolet light protection, biochemical transformations (photosynthesis, nitrogen and sulfur fixation, etc), (Taylor et al., 2007; Radax et al., 2012) as well as in bioactive compounds production related to their chemical defence (Moitinho-Silva et al., 2017). Since sponges actively pump large amounts of water to filter feed, with some Porifera that can filter up to 1000 ml of water per second and per 1 ml of sponge (Reiswig, 1971), they are exposed to their environment more than many others (Pérez-Botello and Simões, 2021). This filtering efficiency traps is reported within sponges-bacteria association widely (Schmitt et al., 2012; Versluis et al., 2017; Steffen et al., 2022; Abdelmohsen et al., 2014) as well as within other several aquatic microorganisms, including viruses (Butina et al., 2022; Canuti et al., 2022), fungi (He et al., 2014; Amend et al., 2019), photosynthetic micro-eukaryotes (single-celled green algae, Choanoflagellata, Diatoms, Dinoflagellata etc) (Nascimento-Silva et al., 2022) and marine mite (Otto, 2000).

However, the identification of WBPP is more problematic as generally their (oo) cysts are known for their resistance to typical aquatic physicochemical degradation and disinfection procedures. As a result, these protozoa can survive and remain in water during multi-barrier water treatment methods (Efstratiou et al., 2017; Karanis, 2018) and hence be involved in waterborne outbreaks worldwide (Ma et al., 2022). Given the environmental resilience of the WBPP, the fecal-oral transmission route of identified protozoa and the rely of the State of Qatar on coastal seawater desalination as the only drinking water resource (Edmonds et al., 2021), red flags for public health risks are raised. The *Blastocystis* sp. parasite was identified from coastal locations within hyperarid mangrove vicinity and hypersaline shallow subtidal. Despite the absence of coastal industry, farming and livestock wandering within these areas, regular anthropological activities as water sports and group

kayaking are however frequently observed. Even with the identification of *D. fragilis* from different location like the offshores sites, far away from coastal urban centres and their potential polluting inputs, concerns are raised as the oyster beds as well as the most fishing stock in this region are within offshore sites (Al Maslamani et al., 2018). However, fishing is mainly achieved through traditional boats without appropriate human discharge facilities after the fisherman errancy in sea for days before returning to the land with the fish collection.

Hence with the spread of WBPP identification, the regions with the contaminated sponges seem to be under anthropological pressure and the hypothesis of human contamination of the environment can be speculated. Further investigations are therefore needed for the direction of the transmission route between humans and environment to be established. Additionally, the geographic location of the country within the Persian/Arabian Gulf is even more challenging since the Gulf is a shallow, semi-enclosed sea with limited freshwater input and restricted circulation, making it naturally exposed to extreme conditions of temperature and salinity (Al-Khayat and Giraldes, 2020; Fawzi et al., 2022). Consequently, the identification of *Blastocystis* sp. and *D. fragilis* within coastal and offshore sites at different temporal points in our study emphasizes the need to improve public policies regarding sewage management. Identifying human-prevalent and pathogenic subtypes within the current work highlights the probability of waterborne transmission of these parasites within the studied region. These are additional evidence that the local overseeing of the protozoan contamination/transmission may require a One Health and ecological approach.

5. Conclusions

In conclusion, this study confirms the effectiveness of using marine sponges as natural traps for pathogens screening within different ecosystems. The molecular screening and characterization of the WBPP in coastal and offshore sites suggests potential health hazards and sources of contamination. Further investigations are needed to comprehensively understand the diversity and ecological roles of parasites within sponge holobionts and to assess their impact on human and animal health.

CRediT authorship contribution statement

Sonia Boughattas: Conceptualization, Data curation, Writing – original draft. **Albandari Al-Khater:** Data curation. **Dana Albatesh:** Data curation. **Bruno W Giraldes:** . **Marawan Abu-Madi:** Methodology. **Asma A. Althani:** Conceptualization, Funding acquisition. **Fatiha M. Benslimane:** Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

THE DATA ARE SHARED WITHIN THE MS

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