Marine Pollution Bulletin 117 (2017) 197–202

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

First report of Tunisian coastal water contamination by protozoan parasites using mollusk bivalves as biological indicators

Khemissa Ghozzi [a,b], Marianna Marangi [c], Roberto Papini [d], Ibtissem Lahmar [a], Rafika Challouf [b], Najoua Houas [a], Rym Ben Dhiab [b], Giovanni Normanno [c], Hamouda Babba [a], Annunziata Giangaspero [c,*]

[a] Laboratoire de Parasitologie-Mycologie Médicale et Moléculaire (code LR12ES08), Faculté de Pharmacie de Monastir, Université de Monastir, Monastir, Tunisia
[b] Laboratoire de Biodiversité et de Biotechnologie Marine, Institut National des Sciences et Technologies de la Mer, Monastir, Tunisia
[c] Department of Science of Agriculture, Food and Environment, University of Foggia, 71121 Foggia, Italy
[d] Department of Veterinary Sciences, University of Pisa, 56120 Pisa, Italy

ARTICLE INFO

Article history:
Received 5 December 2016
Received in revised form 23 January 2017
Accepted 25 January 2017
Available online 4 February 2017

Keyword:
Bivalve mollusks
Cryptosporidium spp.
Toxoplasma gondii
Giardia duodenalis
Cyclospora cayetanensis
*q*PCR
Tunisia

ABSTRACT

In order to establish seawater contamination by emerging protozoan parasites, we used *q*PCR to molecularly characterize and evaluate the parasitic burden of *Giardia duodenalis*, *Cryptosporidium* spp., *Toxoplasma gondii*, and *Cyclospora cayetanensis* in 1255 wild bivalve mollusks collected along the Tunisian coasts. *T. gondii*, *G. duodenalis* and *C. cayetanensis* were detected in 6.9% (99% CI = 1.6–12.2%) pools of *Ruditapes decussatus*. None of the samples were found positive to *Cryptosporidium* spp.; 6.6% pools of *R. decussatus* were positive for *T. gondii* Type I, 1.6% for *G. duodenalis* assemblage A, and 1.6% for the association *T. gondii* Type I/*C. cayetanensis*/*G. duodenalis* assemblage A. *R. decussatus* harbored up to 77500 oocysts/sample of *T. gondii*, up to 395 cysts/sample of *G. duodenalis*, and 526 oocysts/sample of *C. cayetanensis*. These results provide the first evidence that the Tunisian coasts are contaminated by zoonotic protozoan parasites that can constitute a direct or indirect risk for human health.

1. Introduction

Worldwide, authorities responsible for protecting the natural environment and human health are increasingly interested in seawater quality. The ecological pressure caused by intense anthropogenic activities means that all the world's marine environments, including shallow coastal areas, river estuaries and water basins, are considered a kind of "*litmus test*" because they may be contaminated by runoff from agricultural, suburban and urban land and by wastewater discharges. Coastal ecosystems are threatened by the improper disposal of sewage, inefficient/non-functioning treatment plants, illegal wastewater discharge, artificial and natural runoff, and a wide range of industrial waste products (Clark, 2001; Barhoumi et al., 2014). Feces from humans, their pets, domesticated and wild animals may carry numerous pathogenic microorganisms, which can easily contaminate the sea and its inhabitants when released into estuaries and marine environments worldwide (Adell et al., 2014; Giangaspero et al., 2014).

Able to filter large volumes of water and to retain and concentrate microorganisms, shellfish are considered the best biological indicators of the health conditions of marine environments (Chaffai, 2014; Zuykov et al., 2013) They are used extensively worldwide to monitor pollution in aquatic environments, thereby saving time, labor, and money, in comparison with water analysis (Palos Ladeiro et al., 2014).

Shellfish can accumulate several pathogens of anthroponotic and/or zoonotic origin, and those which cause most concern are viruses (e.g., Norovirus, Hepatitis A virus) and bacteria (e.g., pathogenic *Escherichia coli*, *Campylobacter jejuni*, *Salmonella* spp., *Vibrio vulnificus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*), but also protozoan parasites (including *Cryptosporidium*, *Cyclospora*, *Giardia* and *Toxoplasma*). Detection of these pathogens in shellfish indicates fecal contamination of seawater, provides a good indication of the biological pollution of marine ecosystems, and thus identifies possible risks for human health.

Worldwide, *Cryptosporidium* spp., *Giardia duodenalis*, *Cyclospora cayetanensis* and *Toxoplasma gondii* have been detected in farmed or wild shellfish in lagoons and other marine environments (reviewed by Robertson, 2007). Along the Mediterranean Sea coasts, several edible and inedible shellfish have been found to carry protozoan pathogens, either alone and/or in association. These include *Cryptosporidium* spp. in Spain, Portugal and Italy (Giangaspero et al., 2005, 2014; Gomez-Couso et al., 2006; Melo et al., 2006); *G. duodenalis* in Spain and Italy (Gómez-Couso et al., 2005; Giangaspero et al., 2014), *Toxoplasma* in Turkey and Italy (Putignani et al., 2011; Aksoy et al., 2014), and *Cyclospora* in Turkey (Aksoy et al., 2014).

Tunisia's coastline is 1300 km long; the northern coast is fringed by short floodplains with lagoons and coastal lakes, whereas the southern

* Corresponding author.
E-mail address: annunziata.giangaspero@unifg.it (A. Giangaspero).

http://dx.doi.org/10.1016/j.marpolbul.2017.01.057
0025-326X/

coast is low and has large bays into which several rivers and watercourses flow. Fishing activities, aquaculture and shellfish farms are very widespread along the Tunisian coast and in saltwater catchment areas (FAO, 2015).

The presence of enteric viruses in Tunisia has been documented in sewage samples and in shellfish tissues (Elamri et al., 2006; Sdiri-Loulizi et al., 2010); whereas the presence of protozoan parasites has been recorded in sludge and wastewater samples (Ben Ayed et al., 2009, 2012).

These studies highlight the need to protect public health, and suggest that seawater may be subject to fecal contamination. However, no research has yet investigated biological contamination with pathogenic protozoa along the Tunisian coasts. In this context, we aimed to provide a first evaluation of fecal pollution of Tunisian coastal waters via molecular detection and characterization of some important intestinal protozoan parasites (i.e., *G. duodenalis*, *Cryptosporidium* spp., *C. cayetanensis* and *T. gondii*), using wild shellfish found along the Tunisian coastline as environmental biosentinels.

2. Materials and methods

2.1. Study area

Four Tunisian coastal areas were investigated, from North to South: Bizerte Lagoon, Monastir Bay, Chebba and the Gulf of Gabès (Fig. 1).

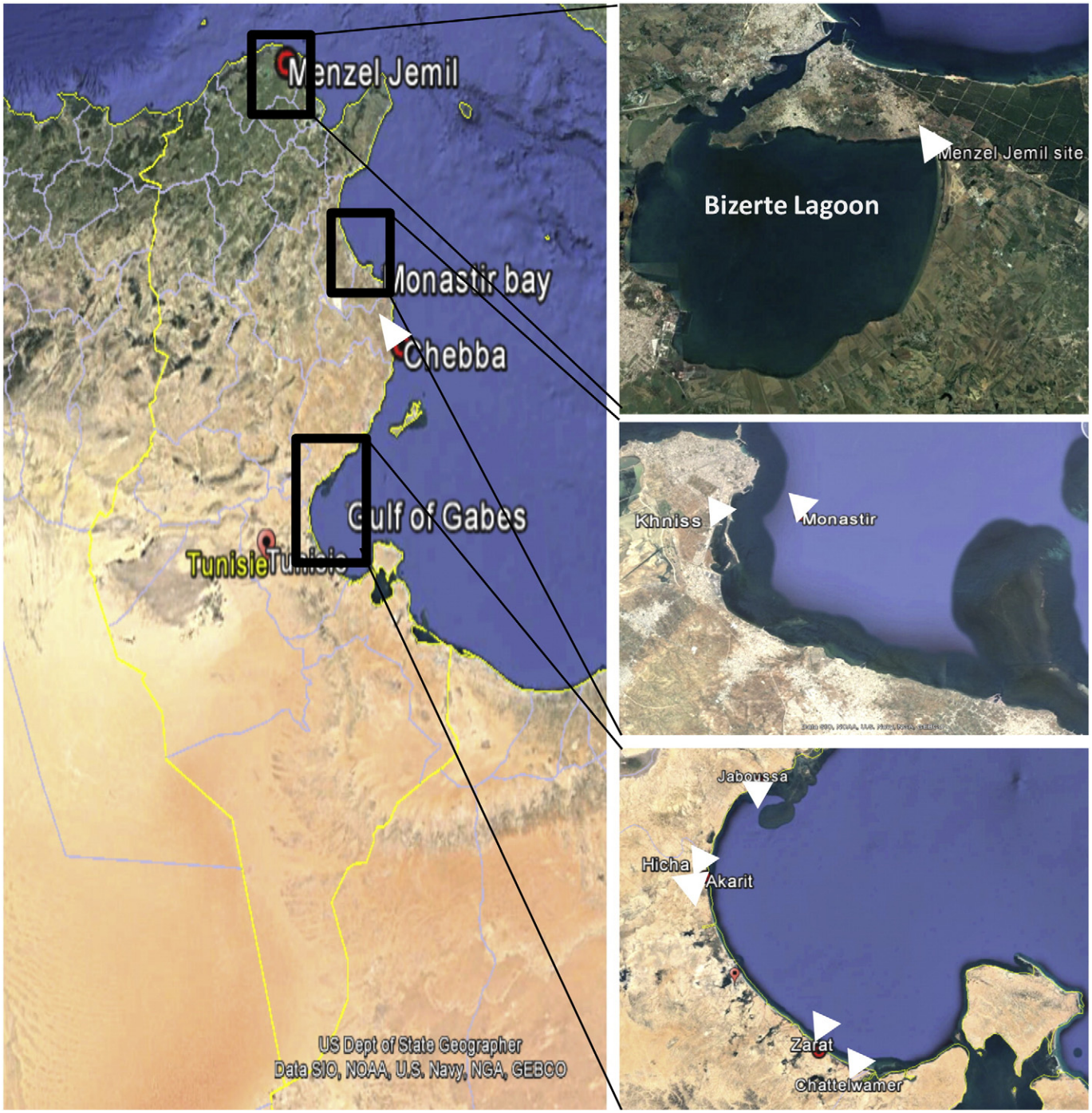

Fig. 1. Map of collection sites on Tunisia coasts. Sites are indicated with white triangles.

Bizerte Lagoon (37°08'-37°146' N/9°48'-9°56'E) is on the northern coast of Tunisia in the Governorate of Bizerte. It has a surface area of 121.6 km^2, a volume of 851.2 × 10^6 m^3 and an average depth of 8 m (Sakka Hlaili et al., 2008). This lagoon has used for fishing for years and hosts intensive shellfish farming activities, including Mediterranean mussel (*Mytilus galloprovincialis*), European flat oyster (*Ostrea edulis*), and Japanese oyster (*Crassostrea gigas*). There are also natural beds of the European clam (*Ruditapes decussatus*) in the lagoon (Alves Martins et al., 2015).

Monastir Bay (35°47′-35°37′N/10°45′E-11°50′E) is in the central coastal area of Tunisia, in the Governorate of Monastir. It is a wide bay with a surface area of over 70 km^2 and is no >3 m deep up to 2 km from the shoreline (Jebali et al., 2011). Only fish farms are present in the bay, but wild shellfish can be found attached to the cages used for fish farming: Mediterranean mussel (*M. galloprovincialis*), brown mussel (*Perna perna*) and Gulf pearly-oyster (*Pinctada radiata*). In addition, grooved carpet shell clam (*R. decussatus*) is naturally present in the bay, together with other bivalve species: fan mussel (*Pinna nobilis*), white furrow shell (*Abra alba*), lagoon cockle (*Cerastoderma glaucum*) and Mediterranean awning clam (*Solemya togata*) (Zamouri-Langar et al., 2011).

Chebba (35°13' N/11°09' E) is in the central-southern area of Tunisia, in the Governorate of Mahdia, where over 200 ha area used for intensive farming of blue fish. There are no shellfish farms, but *R. decussatus*, fan mussel (*P. nobilis*) and Gulf pearly-oyster (*P. radiata*) are the commonest wild species found along this stretch of coast (Rabaoui et al., 2013).

The Gulf of Gabès (33°50'- 34°46' N/10°44'- 11°20' E) is on the southern coast of Tunisia, bounded by the Kerkennah Islands to the northeast and by Djerba Island to the southeast. There are no shellfish farms here, but there are some offshore fish farms. The most abundant and widespread shellfish in this area are grooved carpet shell clam (*R. decussatus*), Gulf pearly-oyster (*P. radiata*), lagoon cockle (*C. glaucum*), fan mussel (*M. galloprovincialis*), and endosymbiont-bearing clam *Loripes lacteus* (Ben Abdallah et al., 2006; Derbali, 2011).

2.2. Sampling and processing

From October 2013 to January 2016, a total number of 1255 wild shellfish belonging to four bivalve mollusk species were sampled. These included *R. decussatus* (n = 1020), *P. radiata* (n = 135), *M. galloprovincialis* (n = 54) and *P. perna* (n = 46). They were collected in the most easily accessible areas corresponding to estuary effluents/rivers and/or natural or artificial watercourses at nine sites: Menzel Jemil (Bizerte Lagoon), Monastir Bay and Khniss (Monastir Bay), Chebba (Mahdia), and Jaboussa, Hicha, Zarrat, Akarit and Chatt Elwamer (Gulf of Gabès) (Fig. 1, Table 1).

After identification *in situ*, specimens were hand-collected from the sand (*R. decussatus*) and fish cages (*P. radiata*, *M. galloprovincialis*, *P. perna*).

After collection, specimens were kept at 0–5 °C until arrival at the laboratory, where they were pooled. Depending on the size and the species of specimens, each sample was composed of a pool of 9–18 specimens of mussels, clams or oysters. Sample numbers and collection sites are shown in Table 1. For each pool, all mollusk flesh was aseptically removed and placed in phosphate-buffered saline (PBS) 0.04 M (pH 7.2–7.4), then sieved using a double layer of gauze and centrifuged for 10 min at 1000*g*, followed by aspiration of the supernatant. The pellet was washed twice with TE buffer (1000*g*, 4 °C, 10 min and then 13,000*g*, 4 °C, 15 min). 500 μl of the pellet was subjected to three freeze/thaw cycles (−80 °C/+80 °C for 5 min) and then DNA was extracted using Qiagen mini kit tissue protocol (Qiagen, Hilden, Germany) according to the manufacture's protocol. Genomic DNA was stored at −20 °C until molecular analysis.

2.3. qPCR

The *q*PCR was performed as described in Marangi et al. (2015) using CFX-96 Real Time Instrument (BioRad, Italy). Briefly, PCRs were carried out in a final volume of 20 μl, using SsoFast™EvaGreen® Supermix (cat. no. 172-5201; Bio-Rad, Italy) and 0.5 μM of species specific primers for B1 *T. gondii* locus (TOXB41-F: 5′-CGAAGCTGAGATGCTCAAAGTC-3′ and TOXB169-R: 5′-AATCCACGTCTGGGAAGAACTC-3′) (Burg et al., 1989); beta giardin *G. duodenalis* gene (GGL-F: 5′-AGTGCGTCAACGAGCAGCT-3′ and GGR-R: 5′-AGTGCTTTGTGACCATCGA-3′) (Holberton and Marshall, 1995); ITS-2 *C. cayatanensis* gene (CCITS2-F: 5′-GCAGTCAC AGGAGGCATATATCC-3′ and CCITS2-R:5′-ATGAGAGACCTCACAGCCA AAC-3′) (Olivier et al., 2001) and COWP *C. parvum* gene (CRYINT2D-F: 5′-TTTGTTGAAGARGGAAATAGATGTG-3′ and CRY2D-R: 5′-GGACKGA AATRCAGGCATTATCYTG-3′) (Ranucci et al., 1993).

Genomic DNA, the positive control for the four parasites, and the negative control (ultrapure Millipore water) in 5 μl was added to the reaction. PCR cycling conditions were as follows: initial denaturation at 98 °C for 2 min, followed by 40 cycles at 98 °C for 5 s, and 15 s at 62 °C (*T. gondii*), 59 °C (*C. cayetanensis*), 61 °C (*G. duodenalis*) and

Table 1
Distribution of the number (no.), prevalence (%), 99% confidence interval (99% CI), and total number of pools of marine bivalve mollusks collected along Tunisian coasts, examined by *q*PCR, and found contaminated by *Cryptosporidium* spp., *Toxoplasma gondii* Type I, *Giardia duodenalis* assemblage A, or their association with *Cyclospora cayetanensis*, according to geographical areas and sites of collection along with species examined and total number of specimens, number of pools, and number of specimens pooled for each species.

Geographical areas	Sites	Species examined (no. of specimens)	No. examined pools	No. positive pools (%, 99% CI)				Total positive no. (%, 99% CI)
				Cryptosporidium spp.	*T. gondii* Type I	*G. duodenalis* assemblage A	*T. gondii* Type I/ *C. cayetanensis*/ *G. duodenalis* assemblage A	
North	Bizerte Lagoon	*Ruditapes decussatus* (n = 138)	9	0	0 (0%, 0–0%)	0 (0%, 0–0%)	0 (0%, 0–0%)	0 (0%, 0–0%)
Centre North	Monastir Bay	*Mytilus galloprovincialis* (n = 54)	6	0	0 (0%, 0–0%)	0 (0%, 0–0%)	0 (0%, 0–0%)	0 (0%, 0–0%)
		Perna perna (n = 46)	5	0	0 (0%, 0–0%)	0 (0%, 0–0%)	0 (0%, 0–0%)	0 (0%, 0–0%)
		Pinctada radiata (n = 135)	15	0	0 (0%, 0–0%)	0 (0%, 0–0%)	0 (0%, 0–0%)	0 (0%, 0–0%)
		Ruditapes decussatus (n = 504)	28	0	3 (10.7%, 0–25.7%)	1 (3.6%, 0–12.6%)	1 (3.6%, 0–12.6%)	5 (17.9%, 0–36.5%)
Centre South	Chebba	*Ruditapes decussatus* (n = 108)	6	0	0 (0%, 0–0%)	0 (0%, 0–0%)	0 (0%, 0–0%)	0 (0%, 0–0%)
South	Gulf of Gabès	*Ruditapes decussatus* (n = 270)	18	0	1 (5.6%, 0–19.5%)	0 (0%, 0–0%)	0 (0%, 0–0%)	1 (5.6%, 0–19.5%)

50 °C (*Cryptosporidium* spp.). Fluorescence data were collected at the end of each cycle as a single acquisition.

The melting analysis was performed at the end of each PCR run (70 °C to 95 °C at 0.5 °C/5 s). Each sample was run in duplicate, and the amplification cycle threshold (*Ct*) and melting temperature (*Tm*) mean values were calculated. The criteria used to define a positive sample were (a) a detectable amplification curves, (b) a *Tm* value equal to the *Tm* value of one of the four positive controls, and (c) a dF/dT fluorescence value of >2.

2.4. Quantitative analysis

Absolute quantification was performed for the positive samples; the amount of DNA (copies/μl) was calculated by relating the *Ct* mean value of each positive sample to a standard curve obtained from a positive control for *T. gondii*, *C. cayetanensis* and *G. duodenalis*. Moreover, the number of oo/cysts was calculated for *T. gondii* according to Lass et al. (2012), for *C. cayetanensis* according to Varma et al. (2003) and for *G. duodenalis* according to Erlandsen and Rasch (1994).

2.5. Sequencing

Samples positive to *T. gondii*, *C. cayetanensis* and/or *G. duodenalis* were purified using EXO I and FASTAP enzymes (Thermo Fisher Scientific, Netherlands) according to the manufacturer's protocol. Purified PCR products were sequenced in both directions using the Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, Stati Uniti) according to the manufacturer's instructions, and using the same primers as the respective PCR reactions. An ABI PRISM 3130 Genetic Analyzer (Applied Biosystem, Foster City, California, Stati Uniti) was used to determine sequences, electropherograms were inspected by eye, and consensus sequences were determined. Each sequence was compared to the nucleotide sequences available in publicly accessible databases using BLASTn (https://www.ncbi.nlm.nih.gov/blast/) software, and subsequently aligned using the ClustalW program (BioEdit software v.7.2.5).

2.6. Statistical analysis

Positivity rates were calculated as the number of pools testing positive for at least one protozoan species / number of examined pools × 100 with corresponding 99% confidence intervals (99% CI). Range, mean (x̄), and standard error of the mean (SEM) were also determined for oocyst and cyst numbers. Statistical analysis was performed using commercial software (GraphPad Prism 7, USA).

3. Results

Molecular techniques revealed that six out of 87 (6.9%, 99% CI = 1.6–12.2%) pools of marine bivalve mollusks were contaminated with at least one intestinal protozoan species. All positive pools consisted of clam species *R. decussatus*. In particular, 4/61 (6.6%, 0.3–12.8%) pools of *R. decussatus* were positive for *T. gondii* Type I, 1 (1.6%, 0–4.8%) for *G. duodenalis* assemblage A, and 1 (1.6%, 0–4.8%) for the association *T. gondii* Type I/*C. cayetanensis*/*G. duodenalis* assemblage A. No samples tested positive for *Cryptosporidium* spp.

All contaminated pools came from Khniss (Monastir Bay), except for one *Toxoplasma* positive pool from Jaboussa (Gulf of Gabès); these accounted for 5/28 (17.9%, 3.7–32%) and 1/18 (5.6%, 0–16.1%) positivity rates in pools of *R. decussatus* collected at these sites, respectively (Table 1). Therefore, 1/2 sampling sites in Monastir Bay was found to be contaminated with fecal protozoa, and 1/5 in the Gulf of Gabès.

Contaminated batches of *R. decussatus* harbored 526 oocysts of *C. cayetanensis* per sample, 62 and 395 (x̄ = 228.5, SEM = 166.5) cysts of *G. duodenalis* per sample, and between 1250 and 77,500 (x̄ = 24,694, SEM = 14,254.5) oocysts of *T. gondii* per sample (Table 2).

Table 2
Number of oocysts/sample of *Toxoplasma gondii* Type I and *Cyclospora cayetanensis* or cysts of *Giardia duodenalis* assemblage A in six batches of clams *Ruditapes decussatus* collected at the coastal areas of Monastir Bay and Gulf of Gabès in Tunisia, examined by *q*PCR.

Site of collection	Sample identification	Protozoans		
		T. gondii Type I	*C. cayetanensis*	*G. duodenalis* assemblage A
Monastir Bay (Khniss)	Pf2	32,000	526	62
	PM3	6200	0	0
	PS1	6520	0	0
	PN2	0	0	395
	Pd1	77,500	0	0
Gulf of Gabès (Jaboussa)	JO1	1250	0	0

Since the present oocyst/cyst counts were obtained from pooled shellfish, it is very likely that wide variations in oocyst/cyst content occurred between individual bivalves.

The nucleotide sequences have been deposited in the GenBank database under accession numbers **KY510286-KY510291** (*T. gondii*), **KY510283-KY510284** (*G. duodenalis*) and KY510285 (*C. cayetanensis*).

4. Discussion

This is the first environmental study investigating fecal contamination by protozoan parasites along the Tunisian coasts, and the first report of *T. gondii*, *C. cayetanesis* and *G. duodenalis* in shellfish from North Africa.

Two of the nine investigated coastal sites harbored protozoan parasites; one in Monastir Bay (Khniss, central Tunisian coastline) and one in the Gulf of Gabès (Jaboussa, southern coast), with percentages of 17.9% and 5.6% respectively. Protozoans were detected in 6.9% of the investigated marine bivalve mollusks, and *R. decussatus* was the only species contaminated by *T. gondii*, *G. duodenalis*, and *C. cayetanensis* alone, or in association (*T. gondii*/*C. cayetanensis*/*G. duodenalis*).

In the Mediterranean countries, *T. gondii* has been reported in edible shellfish in Italy, i.e. farmed oyster (*Crassostrea gigas*) and clam (*R. decussatus*) (Putignani et al., 2011), and also in mussel (*M. galloprovincialis*) in Turkey (Aksoy et al., 2014). *G. duodenalis* has been detected in clams (*Chamelea gallina*) (Giangaspero et al., 2004) and mussel *M. galloprovincialis* farmed along the Italian Adriatic coast and on sale (Giangaspero et al., 2014), and in *M. galloprovincialis* in Spain (Gómez-Couso et al., 2005). In addition, *C. cayetanensis* was first recorded in Turkey in *M. galloprovincialis* (Aksoy et al., 2014).

Here, *T. gondii* Type I - the most pathogenic lineage (Robert-Gangneux and Dardé, 2012) and with restricted host range (Herrmann et al., 2014) - was identified in 5.7% of *R. decussatus* (alone or in association with *G. duodenalis* and *C. cayetanensis*) from Khniss (Monastir Bay) and Jaboussa (Gulf of Gabès) with a very high number of oocysts (up to 77,500/batch).

In Tunisia, the level of human seroprevalence for toxoplasmosis is high, and Types I, and I/II and I/III have been recorded in humans (Boughattas et al., 2010). A high level of seroprevalence has also been registered in sheep (Boughattas et al., 2014), goats (Amairia et al., 2016), cattle (Lahmar et al., 2015), horses (Boughattas et al., 2011) and poultry (Boughattas and Bouratbine, 2014, 2015). It has also been shown that sheep harbor type I, II and III (Boughattas et al., 2014) and chickens Type II (Boughattas and Bouratbine, 2015).

The very high level of environmental contamination by *Toxoplasma* oocysts in Tunisia is due to the great number of cats in the country, and there are no government programs to limit the numbers of the stray animals.

G. duodenalis Assemblage A - the most zoonotic assemblage (Ryan and Cacciò, 2013) - was detected in 22.3% of *R. decussatus* (alone or in association with *G. duodenalis* and *C. cayetanensis*) from one site

(Khniss, Monastir Bay) with a cyst burden number of up to 395 cysts/batch. No data on *G. duodenalis* in animals are available for Tunisia, but in the country, this protozoan is highly prevalent in children (Gharbi et al., 1999), food handlers (Siala et al., 2011) and in symptomatic patients (Bouratbine et al., 2000). Whereas assemblages circulating in humans remain unknown, *G. duodenalis* assemblages AI, AII, B and E were detected in treatment plants (Ben Ayed et al., 2009, 2012).

This study revealed also the presence of *C. cayetanensis* in 1.1% of *R. decussatus* in association with *T. gondii* and *G. duodenalis*, and from one site (Khniss, Monastir Bay) with a burden number of 526 oocysts/batch.

C. cayetanensis is primarily regarded as a cause of traveler's diarrhea, but no data are available on the diffusion of this parasite in humans in Tunisia, as it is not usually included in routine laboratory investigations. The low prevalence detected in this study is consistent with that registered in wastewater samples (Ben Ayed et al., 2012).

In this study, the failure to detect *Cryptosporidium* is actually quite unexpected, considering that in Tunisia *Cryptosporidium* is a common protozoan parasite in humans with diarrhea, in immune-compromised patients (Bouratbine et al., 2000) and in wastewater (Ben Ayed et al., 2012). However, its presence in Tunisia is limited to a few animal host species, such as calves (Soltane et al., 2007a, Rahmouni et al., 2014), sheep, chickens (Soltane et al., 2007b); cattle populations are the main causes of environmental contamination with *Cryptosporidium* oocysts at other latitudes (Xiao and Feng, 2008), but calves farming are confined to a geographically limited area of Tunisia (Rahmouni et al., 2014).

In this study, *T. gondii* was much more common than both *G. duodenalis* and *C. cayetanensis* in wild clams, and there was a significant difference in the percentage of test-positive samples between geographical sites. These findings are not entirely unexpected. Possible explanations for a higher level of oocyst/cysts contamination in Monastir Bay (Khniss) and the Gulf of Gabès (Jaboussa) may be related to the known presence of effluents from treatment plants and agricultural and urban activities in these areas (Jebali et al., 2011). In particular, Khniss is situated in a marine depression, where intense pollution causes great environmental problems (Sellami, 2015), and both Monastir Bay and the Gulf of Gabès have shallow waters with sandbanks that reduce the effects of wave movement (Nouira et al., 2013).

The negative results we obtained at the northern site (i.e. Bizerte lagoon) might be related to the predominance of industrial waste, i.e., pesticide and chemical fertilizer wastes in the investigated area (which have caused a decline in aquaculture in recent years) (Barhoumi et al., 2014) rather than to agricultural or urban waste. Alternatively, this might be related to the presence of more efficient treatment plants in the selected area (i.e. Menzel Jemil). Moreover, it is possible that the environmental characteristics of Bizerte lagoon, i.e. the continuous changes in temperature, salinity and pH caused by sea currents from the Mediterranean Sea and fresh water currents from the Tinja River (Barhoumi et al., 2014), may negatively influence the availability and viability of the investigated protozoans.

Investigated protozoan pathogens can persist for long periods of time in the environment, in fresh or saltwater, and in shellfish; it is well known that they maintain their infectivity (Lindsay and Dubey, 2009; Freire-Santos et al., 2000; Ortega et al., 1998) and the infectious dose is low (Robertson, 2007). Thus, recreational activities along the coasts and consumption (internal or after exportation) of shellfish should be considered as risk factors for people in Tunisia (including tourists). *R. decussatus* is Tunisia's most widespread shellfish, and is an important economic endpoint because it is a natural resource (Chaffai, 2014). Since it was the only contaminated shellfish species found in the present study, *R. decussatus* can be used as a biosentinel of protozoa contamination in seawater environments.

In conclusion, the findings of the present study indicate that wild shellfish collected along Tunisia's coasts harbor genomic DNA of *T. gondi* Type I, *G. duodenalis* assemblage A and *C. cayetanensis*. This gives cause for concern about public health, because it means that Tunisian coastal waters constitute a risk, both directly (through bathing or recreational activities on the beaches or inflowing water courses) and indirectly (through the consumption of raw or undercooked bivalves). Thus, the Tunisian public health authorities should promote strict control measures and careful monitoring procedures in order to prevent seawater contamination.

Declaration of interest

The Authors declare that they have no conflict of interest.

Contributors

KG, IL and NH conceived the sample collection; KG, RC and RB collected samples; MM, AG, and GN conceived the molecular study; MM and KG performed the molecular studies; RP performed the statistical analysis; AG, KG, MM, RP and HN drafted the paper, and all authors contributed to editing the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

The authors wish to thank Tiziana Tedde and Tiziana Caradonna for their helpful work in the lab.

The present work was partially funded by L.A.I.F.F. Project (codice n. 47); "PO Puglia FESR-2007-2013, Asse I, Linea 1.2. Accordo di Programma Quadro in materia di Ricerca Scientifica. Intervento "Reti di Laboratori Pubblici di Ricerca" (Responsible: Annunziata Giangaspero).

References

Adell, A.D., Smith, W.A., Shapiro, K., Melli, A., Conrada, P.A., 2014. Molecular epidemiology of *Cryptosporidium* spp. and *Giardia* spp. in mussels (*Mytilus californianus*) and California sea lions (*Zalophus californianus*) from Central California. Appl. Environ. Microbiol. 80, 7732–7740.

Aksoy, U., Marangi, M., Papini, R., Ozkoc, S., Bayram Delibas, S., Giangaspero, A., 2014. Detection of *Toxoplasma gondii* and *Cyclospora cayetanensis* in *Mytilus galloprovincialis* from Izmir Province coast (Turkey) by real time PCR/high resolution melting analysis (HRM). Food Microbiol. 44, 128–135.

Alves Martins, M.V., Zaaboub, N., Aleya, L., Frontalini, F., Pereira, E., Miranda, P., et al., 2015. Environmental quality assessment of Bizerte lagoon (Tunisia) using living foraminifera assemblages and a multiproxy approach. PLoS One 10, e0137250. http://dx.doi.org/10.1371/journal.pone.0137250.

Amairia, S., Rouatbi, M., Rjeibi, M.R., Nouasri, H., Sassi, L., Mhadhbi, M., Gharbi, M., 2016. Molecular prevalence of *Toxoplasma gondii* DNA in goats' milk and seroprevalence in Northwest Tunisia. Vet. Med. Sci. 2, 154–160.

Barhoumi, B., Le Menach, K., Devier, M.H., Ben Ameur, W., Etcheber, H., Budzinski, H., Cachot, J., Driss, M.R., 2014. Polycyclic aromatic hydrocarbons (PAHs) in surface sediments from the Bizerte Lagoon, Tunisia: levels, sources, and toxicological significance. Environ. Monit. Assess. 186, 2653–2669.

Ben Abdallah, O., Ben Hajhamida, N., Jarboui, O., 2006. Evaluation et cartographie de coquillages dans la partie e marine de la région de zarzis. Bull. Inst. Natn. Scien. Tech. Mer de Salammbô. 33.

Ben Ayed, L., Schijven, J., Alouini, Z., Jemli, M., Sabbahi, S., 2009. Presence of parasitic protozoa and helminth in sewage and efficiency of sewage treatment in Tunisia. Parasitol. Res. 105, 393–400.

Ben Ayed, L., Yang, W., Widmer, G., Cama, V., Ortega, Y., 2012. Survey and genetic characterization of wastewater in Tunisia for *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bieneusi*, *Cyclospora cayetanensis* and *Eimeria* spp. J. Water Health 10, 431–444.

Boughattas, S., Ben-Abdallah, R., Siala, E., Souissi, O., Aoun, K., Bouratbine, A., 2010. Direct genotypic characterization of *Toxoplasma gondii* strains associated with congenital *Toxoplasmosis* in Tunisia (North Africa). Am.J.Trop. Med. Hyg. 82, 1041–1046.

Boughattas, S., Bergaoui, R., Essid, R., Aoun, K., Bouratbine, A., 2011. Seroprevalence of *Toxoplasma gondii* infection among horses in Tunisia. Parasit. Vectors 4, 218.

Boughattas, S., Ayari, K., Sa, T., Aoun, K., Bouratbine, A., 2014. Survey of the parasite *Toxoplasma gondii* in human consumed ovine meat in Tunis City. PLoS One 9, 89–92.

Boughattas, S., Bouratbine, A., 2014. Prevalence of food-borne *Toxoplasma gondii* in free-ranging chickens sold in Tunis, Tunisia. J. Food Qual. Hazards. Control 1, 89–92.

Boughattas, S., Bouratbine, A., 2015. Genetic characterization of *Toxoplasma gondii* isolated from chicken meats in Tunisia. J. Food Qual. Hazards Control 2, 97–100.

Bouratbine, A., Aoun, K., Siala, E., Chahed, M.K., Ben Hassine, L., Meherzi, A., 2000. Pour une meilleure estimation de la prévalence du parasitisme intestinal dans la région de Tunis. Bull. Soc. Pathol. Exot. 93, 353–355.

Burg, J.L., Grover, C.M., Pouletty, P., Boothroyd, J.C., 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. J. Clin. Microbiol. 27, 1787–1792.
Chaffai, A., 2014. Usefulness of bioindicators and biomarkers in pollution biomonitoring. Int. J. Biotech. Well. Indus. 3, 19–26.
Clark, R.B., 2001. Marine Pollution. fifth ed. Clarendon Press, Oxford, p. 237 ISBN 0 19 879292 1, 2001.
Derbali, A., 2011. Biology, abundance and cartography of two bivalves species: the pearl-oyster *Pinctada radiata* and the cockle *Cerastoderma glaucum* in the Gulf of Gabes. Ph.D. Thesis. University of Sfax, Tunisia, p. 169.
Elamri, D.E., Aouni, M., Parnaudeau, S., Le Guyader, F.S., 2006. Detection of human enteric viruses in shellfish collected in Tunisia. Lett. Appl. Microbiol. 43, 399–404.
Erlandsen, S.L., Rasch, E.M., 1994. The DNA content of trophozoites and cysts of *Giardia lamblia* by microdensitometric quantitation of Feulgen staining and examination by laser scanning confocal microscopy. J. Histochem. Cytochem. 42, 1413–1416.
FAO, 2015. National Aquaculture Sector Overview Tunisia. Fisheries and Aquaculture Department.
Freire-Santos, F., Oteiza-Lopez, A.M., Vergara-Castiblanco, C.A., Arees Mazas, M.E., Alvarez-Suarez, E., Garcia-Martin, O., 2000. Detection of *Cryptosporidium* oocysts in bivalve molluscs destined for human consumption. J. Parasitol. 86, 853–854.
Gharbi, T., Chaker, E., Boughedir, J., el Mabrouk, S., Ben Rayana, M.C., 1999. Study of anemia in giardiasis intestinalis in Tunisian preschool children. Tunis. Med. 77, 558–561.
Gómez-Couso, H., Mendez-Hermida, F., Castro-Hermida, J.A., Ares-Mazas, E., 2005. *Giardia* in shellfish-farming areas: detection in mussels, river water and wastewaters. Vet. Parasitol. 133, 13–18.
Gomez-Couso, H., Mendez-Hermida, F., Castro-Hermida, J.A., Ares-Maza's, E., 2006. *Cryptosporidium* contamination in harvesting areas of bivalve molluscs. J. Food Prot. 69, 185–190.
Giangaspero, A., Molini, U., Traversa, D., Iorio, R., Ceschia, G., 2004. *Giardia* and *Cryptosporidium* in seawater clams (*Chamelea gallina* and *Ruditapes philippinarum*) of the Adriatic coast of central and northern Italy. Atti XXIII Congresso SOIPA. Parassitologia. 46, p. 153.
Giangaspero, A., Molini, U., Iorio, R., Traversa, D., Paoletti, B., Giansante, C., 2005. *Cryptosporidium parvum* oocysts in seawater clams (*Chamelea gallina*) in Italy. Prev. Vet. Med. 69, 203–212.
Giangaspero, A., Papini, R., Marangi, M., Koehler, A.V., Gasser, R.B., 2014. *Cryptosporidium parvum* genotype IIa and *Giardia duodenalis* assemblage A in *Mytilus galloprovincialis* on sale at local food markets. Int. J. Food Microbiol. 171, 62–67.
Herrmann, D.C., Maksimov, A., Hotop, A., Groß, U., Däubener, W., Liesenfeld, O., Pleyer, U., Conraths, F.J., Schares, G., 2014. Genotyping of samples from German patients with ocular, cerebral and systemic toxoplasmosis reveals a predominance of *Toxoplasma gondii* type II. Int. J. Med. Microbiol. 304, 911–916.
Holberton, D.V., Marshall, J., 1995. Analysis of consensus sequence patterns in *Giardia* cytoskeleton gene promoters. Nucleic Acids Res. 23, 2945–2953.
Jebali, J., Ben-Khedher, S., Ghedira, J., Kamel, N., Boussetta, H., 2011. Integrated assessment of biochemical responses in Mediterranean crab (*Carcinus maenas*) collected from Monastir Bay Tunisia. J. Environ. Sci. 23, 1714–1720.
Lahmar, I., Lachkhem, A., Slama, D., Sakly, W., Haouas, N., 2015. Prevalence of toxoplasmosis in sheep, goats and cattle in southern Tunisia. J. Bacteriol. Parasitol. 6, 245–249.
Lass, A., Pietkiewicz, H., Szostakowska, B., Myjak, P., 2012. The first detection of *Toxoplasma gondii* DNA in environmental fruits and vegetables samples. Eur. J. Clin. Microbiol. Infect. Dis. 31, 1101–1108.
Lindsay, D.S., Dubey, J.P., 2009. Long-term survival of *Toxoplasma gondii* sporulated oocysts in seawater. J. Parasitol. 95, 1019–1020.
Marangi, M., Giangaspero, A., Lacasella, V., Lonigro, A., Gasser, R.B., 2015. Multiplex PCR for the detection and quantification of zoonotic taxa of *Giardia*, *Cryptosporidium* and *Toxoplasma* in wastewater and mussels. Mol. Cell. Probes. 29, 122–125.
Melo, P.C., Teodosio, J., Reis, J., Durate, A., Costa, J.C., Fonseca, I.P., 2006. *Cryptosporidium* spp. in freshwater bivalves in Portugal. J. Eukaryot. Microbiol. 53, S28–S29.
Nouira, T., Risso, C., Lassaad, C., Budzinski, H., Boussetta, H., 2013. Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in surface sediments from Monastir Bay (Tunisia, Central Mediterranean): occurrence, distribution and seasonal variations. Chemosphere. 93, 487–493.
Olivier, C., van de Pas, S., Lepp, P.W., Yoder, K., Relman, D.A., 2001. Sequence variability in the first internal transcribed spacer region within and among *Cyclospora* species is consistent with polyparasitism. Int. J. Parasitol. 31, 1475–1487.
Ortega, Y.R., Sterling, C.R., Gilman, R.H., 1998. Cyclospora cayetanensis. Adv. Parasitol. 40, 399–418.
Palos Ladeiro, M., Aubert, D., Villena, I., Geffard, A., Bigot, A., 2014. Bioaccumulation of human waterborne protozoa by zebra mussel (*Dreissena polymorpha*): interest for water biomonitoring. Water Res. 48, 148–155.
Putignani, L., Mancinelli, L., Del Chierico, F., Menichella, D., Adlerstein, D., Angelici, M., Marangi, M., Berrilli, F., Caffara, M.D., di Regalbono, A.F., Giangaspero, A., 2011. Investigation of *Toxoplasma gondii* presence in farmed shellfish by nested-PCR and real-time PCR fluorescent amplicon generation assay (FLAG). Exp. Parasitol. 127, 409–417.
Rabaoui, L., Balti, R., El Zrelli, R., Tlig-Zouari, S., 2013. Assessment of heavy metal pollution in the gulf of Gabes (Tunisia) using four mollusc species. Mediterr. Mar. Sci. 15, 45–58.
Ranucci, L., Müller, H.M., La Rosa, G., Reckmann, I., Morales, M.A., Spano, F., et al., 1993. Characterization and immunolocalization of a *Cryptosporidium* protein containing repeated amino acid motifs. Infect. Immun. 61, 2347–2356.
Rahmouni, I., Essid, R., Aoun, K., Bouratbine, A., 2014. Glycoprotein 60 diversity in *Cryptosporidium parvum* causing human and cattle cryptosporidiosis in the rural region of Northern Tunisia. Am.J.Trop. Med. Hyg. 90, 346–350.
Robert-Gangneux, F., Dardé, M.L., 2012. Epidemiology of and diagnostic strategies for toxoplasmosis. Clin. Microbiol. Rev. 25, 583.
Robertson, L.J., 2007. The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: a review. Int. J. Food Microbiol. 120, 201–216.
Ryan, U., Cacciò, S.M., 2013. Zoonotic potential of *Giardia*. Int. J. Parasitol. 43, 943–956.
Sakka Hlaili, A., Grami, B., Niquil, N., Gosselin, M., Hamel, D., et al., 2008. The planktonic food web of the Bizerte lagoon (South-Western Mediterranean) during summer: spatial distribution under different anthropogenic pressures. Estuar. Coast. Shelf Sci. 78, 61–77.
Sellami, R., 2015. La baie de Monastir face à la pollution de la frange littorale: l'éveil d'une conscience environnementale locale. Actes du symposium international- Kerkennah (Sfax) Tunisie 20–24 octobre 2015, pp. 275–281 (ISBN: 978-2-9554403-0-8).
Siala, E., Guidara, R., Ben Abdallah, R., Ben Ayed, S., Ben Alaya, N., Zallaga, N., Bouratbine, A., Aoun, K., 2011. The intestinal parasites in the food handlers of Tunis area: study of 8502 stool samples (1998–2008). Arch. Inst. Pasteur Tunis. 88 (1–4), 77–84.
Sdiri-Loulizi, K., et al., 2010. First molecular detection of Aichi virus in sewage and shellfish samples in the Monastir region of Tunisia. Arch. Virol. 155, 1509–1513.
Soltane, R., Guyot, K., Dei Cas, E., Ayadi, A., 2007a. Prevalence of *Cryptosporidium* spp. (Eucoccidiorida: Cryptosporiidae) in seven species of farm animals in Tunisia. Parasite. 11, 335–338.
Soltane, R., Guyot, K., Dei-Cas, E., Ayadi, A., 2007b. *Cryptosporidium parvum* (Eucoccidiorida: Cryptosporiidae) in calves: results of a longitudinal study in a dairy farm in Sfax, Tunisia. Parasite 14, 309–312.
Varma, M., Hester, J.D., Schaefer, F.W., Ware 3rd, M.W., Lindquist, H.D., 2003. Detection of *Cyclospora cayetanensis* using a quantitative real-time PCR assay. J. Microbiol. Methods 53, 27–36.
Xiao, L., Feng, Y., 2008. Zoonotic cryptosporidiosis. FEMS Immunol. Med. Microbiol. 52: 309–323. http://dx.doi.org/10.1111/j.1574-695X.2008.00377.x (309-323).
Zamouri-Langar, N., Chouba, L., Ajjabi Chebil, L., Mrabet, R., El Abed, A., 2011. Les coquillages bivalves des côtes tunisiennes. Institut National des Sciences et Technologies de la Mer: Salammbô. ISBN 978-9938-9512-0-2. 128.
Zuykov, M., Pelletier, E., Harper, D.A.T., 2013. Bivalve mollusks in metal pollution studies: from bioaccumulation to biomonitoring. Chemosphere. 93, 201–208.