Research Article

Molecular prevalence, risk factors and genotypes of *Toxoplasma gondii* DNA in wild marine snails collected from offshore waters in eastern China

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Abstract

Increasing evidence exisits for the role that shellfish play in the epidemiology of *Toxoplasma gondii* in marine environment. However, limited information is available on the level of *T. gondii* infection in wild marine snails, which can play a role in the transmission of *T. gondii* to other marine organisms and humans. In this study, the prevalence of *T. gondii* DNA in wild marine snails collected from three coastal cities in China was determined. Between January 2018 and November 2019, 1,206 wild marine snails were randomly collected and examined for the presence of *T. gondii* DNA using a nested polymerase chain reaction (PCR) targeting *T. gondii B1* gene. The amplified products were genotyped using multilocus PCR-restriction fragment length polymorphism analysis. We also examined whether species of snail, sampling region, sampling season, surface runoff near samplic site, residential water discharge near samplic site, and proximity to livestock farms are associated with the occurrence of *T. gondii* DNA in marine snails. Our results showed that 23 (1.91%) snails were positive for *T. gondii B1* gene. The genotype of two of the 23 *T. gondii* amplicons was consistent with ToxoDB Genotype #9. Multiple logistic regression revealed that surface runoff near the sampling site (P = 0.039, odds ratio [OR] = 3.413, 95% confidence interval [CI]: 1.07-10.94) and residential water discharge near the sampling site (P = 0.021, OR = 3.990, 95%CI: 1.24-12.87) are more likely to be associated with the presence of *T. gondii* DNA in marine snails. The detection of *T. gondii* DNA in marine snails in China highlights the potential impact of the anthropogenic activities on marine organisms and the potential foodborne risk posed to humans with such an important terrestrial pathogen.

Keywords: Marine snails; *Toxoplasma gondii*; Prevalence; Genotyping; Bioindicator; China

Introduction

Recent years have witnessed increasing interest in exploring the factors that can contribute to the introduction of the protozoan *Toxoplasma gondii* into coastal waters (Shapiro et al., 2010; Simon et al., 2013), where they infect marine animals, such as sea otters, bottlenose dolphin and pinnipeds (Bigal et al., 2018; Bachand et al., 2019; Reisfeld et al., 2019; Shapiro et al., 2019). The environmentally resistant oocyst's stage of *T. gondii* is excreted in the feces of the feline definitive host and can remain viable in the soil or water for years (Lindsay and Dubey, 2009; Dubey, 2010; Lélu et al., 2012). Surface runoff may facilitate the transmission of *T. gondii* oocysts from the land to the aquatic environment (Conrad et al., 2005; Miller et al., 2008), where oocysts can be retained by filter feeder marine bivalve shellfish such as oysters and mussels (Coupe et al., 2018; Cong et al., 2019). Marine snails are also filter feeders (i.e., can accumulate *T. gondii* oocysts) and thus can also play a role in the introduction of terrestrial pathogens to marine ecosystems (Krusor et al., 2015).

T. gondii has been detected in oysters and mussels in many countries (Putignani et al., 2011; Aksoy et al., 2014; Cong et al., 2017, 2019; Coupe et al., 2018; Marquis et al., 2019; Monteiro et al., 2019). Ingestion of infected marine shellfish by marine fish, mammals, or humans could ultimately increase the risk of foodborne infection with *T. gondii* (Jones et al., 2009; Chiang et al., 2014). Marine snails are considered delicacies in China and this can put people at risk of *T. gondii* infection if infected marine snails are ingested raw or undercooked . However, information about the role played by marine snails in the introduction of *T. gondii* oocysts into the marine

environment in China remains largely unknown.

In the present study, we investigated the prevalence of and risk factors associated with the presence of *T. gondii* DNA in wild marine snails in China. It is hoped that the obtained data can improve our knowledge of the extent of marine snail contamination with *T. gondii*, which can ultimately improve our capability to control *T. gondii* infection in wild marine mammals and humans.

2. Materials and methods

2.1 Study areas and sampling sites

This study was performed in three cities in Shandong province, eastern China, namely Weihai, Yantai and Rizhao (Fig. 1A). In Weihai (34°23'~38°24'N, 114°48'~122°42'E), wild marine snails were collected from the shallow seas nearby Shuangdao (SD), Xiaoshidao (XSD), Mazigang (MZG) and Putaotan (PTT) (Fig 1B). In Yantai (36°16'~38°23'N, 119°34'~121°57'E), three sites, namely the second bathing beach (SBB), Yangmadao (YMD), and Xiaobeihai (XBH) were selected for collection of marine snails from shallow offshore waters (Fig. 1C). In Rizhao (35°04'~36°04'N, 118°25'~119°39'E), marine snails were collected from two beach combing parks, namely Liujiwan (LJW) and Wanbao (WB), where people harvest seafood on the beach when the tide is ebbing (Fig. 1D). Details of all sampling information are included in the Supplementary file Table S1.

2.2 Sampling of different marine snail species

From January 2018 to November 2019, a total of 1,206 wild marine snails were collected, including 438 *Rapana venosa*, 380 *Monodonta labio* and 388 *Glossaulax didyma*. Following collection, the wild marine snails were stored in cold boxes and transported to the Laboratory of Marine College, Shandong University for processing. Distilled water was used to wash the outer surfaces of the collected snails. Then, all tissues of the snails were removed, grinded using a mortar and placed in cryogenic vials and kept frozen at –80°C until used in DNA extraction.

2.3 Data collection of the environmental factors

Information about the environmental characteristics of the sampling sites were collected by means of personal interviews with local residents, including fishermen and villagers. We explored the correlation between some anthropogenic activities, such as the presence of livestock farms, occurrence of surface runoff and residential water discharge within 500 meters of the sampling site, and the presence of *T. gondii* in the marine snails.

2.4 DNA extraction and nested PCR

A total of 5g mixed tissue of each wild marine snail was subjected to five alternating freeze-thaw cycles (liquid nitrogen for 5 min followed by 80°C for 5 min) and centrifuged at 9,750g for 2 min. Then, E.Z.N.A.® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA) was used to extract the genomic DNA from each sample.

Isolated DNAs were stored frozen at −20°C.

The detection of *T. gondii* DNA in snail's tissue was examined using a nested PCR, which targets *B1* gene of *T. gondii*. In the first reaction, the primers Toxo 1 (5′AGC GTC TCT CTT CAA GCA GCG TA3′) and Toxo 2 (5′ TCC GCA GCG ACT TCT ATC TCT GT3′) were used to amplify a 300 bp fragment. Then, the primers Toxo 3 (5′TGG GAA TGA AAG AGA CGC TAA TGT G3′) and Toxo 4 (5′TTAAAG CGT TCG TGG TCA ACT ATC G 3′) amplified a 155 bp fragment (Yai et al, 2003).

The nested PCR conditions was performed as described previously (Monteiro et al. 2019). Briefly, for the two PCR reactions, buffer (10 mM Tris-HCl, pH 8.5; 500 mM KCl), 50 mM $MgCl_2$, 2 U of Taq DNA polymerase (TAKARA, Japan), 1.5 mM of each dNTP (dATP, dGTP, dCTP and dTTP) were included in the amplification solution. Also, 5 pmol and 4 pmol of each primer were used for the first reaction and the second reaction, respectively. 5 µl of the extracted DNA (0.5–1 ng) from each sample was used in the first reaction and 1 µl of the PCR product from the first reaction, diluted 1:10, was used in the second reaction, with a 25-µl amplification reaction mixture. Each PCR reaction included a positive (*T. gondii* DNA) and negative (water) control samples.

All reactions were performed using a thermal cycler (PTC 200, Bio-RAD). The first reaction of the PCR program included an initial denaturation at 94°C for 3 min, followed by 25 cycles at 94°C for 45 s, 55°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min. The second reaction involved the same PCR program used in the first reaction, except that 35 cycles were used following the initial

denaturation. PCR products were analyzed by electrophoresis in 1.5% agarose gel, visualized using GoldenView™ and photographed using a gel documentation system (UVP GelDoc-ItTM Imaging System, Cambridge, UK).

2.5 Genotyping of T. gondii amplicons

A total of 11 loci including 10 nuclear loci (i.e., SAG1, alternative SAG2, 5′-and 3′-SAG2, SAG3, L358, BTUB, c22–8, GRA6, c29-2, PK1) and an apicoplast locus Apico were used to genotype the *B1* gene-positive samples using a multilocus PCR-restriction fragment length polymorphism (PCR-RFLP) approach as described previously (Su et al., 2010; Gerhold et al., 2017； Cong et al., 2020; Su and Dubey, 2020). The following strains GT1, PTG, CTG, MAS, TgCgCa1, TgCatBr5, TgCatBr64 and TgRsCr1were used as positive controls (Table 3). Details about the oligonucleotide primers used in the multiplex and nested PCR-based genotyping analysis are listed in Table S2 and Table S3, respectively. The enzyme reaction conditions implemented in the RFLP analysis are shown in Table S4.

2.6. Statistical analysis

Chi-square test was used to examine univariate associations between *T. gondii* prevalence in marine snails and categorical risk factors (e.g. snail species, geographic region, sampling season, surface runoff, residential water discharge and proximity to livestock farms). *P* values <0.05 were considered statistically significant. Then, logistic regression was used to analyze the association between *T. gondii* infection and

the aforementioned factors. Adjusted odds ratios (OR) and 95% confidence interval (CI) were calculated to measure the strength of association between each risk factor and the presence of *T. gondii* DNA in the snail. *P*-values < 0.05 were deemed statistically significant. All the statistical analysis was performed using the SPSS 19.0 software package (IBM, Armonk, NY, United States).

3. Results

Twenty-three (1.9%) samples out of 1,206 wild marine snails were positive for *T. gondii B1* gene. Evidence of *T. gondii* DNA was detected in 13 *Rapana venosa* and 10 *Glossaulax didyma*. No *T. gondii* DNA was detected in *Monodonta labio* (Table 1). Marine snails collected from Yantai had the highest *T. gondii* prevalence (2.47%), followed by Weihai (1.83%) and Rizhao (1.48%). The prevalence of *T. gondii* DNA detected in the snails at different sampling times ranged from 1.02% in Summer to 3.56% in Spring (Table 1). Moreover, environmental features near the sampling sites were also investigated, including surface runoff, residential water discharge and proximity to livestock farms. Results of these environmental features are shown in Table 1.

The univariate analysis revealed only two variables that were associated with the presence of *T. gondii* DNA in wild marine snails, including surface runoff and residential water discharge near the sampling site (Table 1). Further analysis using multivariate logistic regression revealed that surface runoff near the sampling site (*P* = 0.039, OR = 3.413, 95% CI: 1.07-10.94), and residential water discharge nearing

191 the sampling site (P = 0.021, OR = 3.990, 95% CI: 1.24-12.87) were significant and
192 independent risk factor for the presence of *T. gondii* DNA in snails (Table 2).
193 Using PCR-RFLP analysis, two sample out of the 23 positive samples was
194 genotyped and their restriction digest profiles were consistent with that of *T. gondii*
195 ToxoDB#9, which is the most prevalent genotype in China(Table 3).
196

197 **4. Discussion**

198 In the prsent study, we provdied new information on the prevalence of *T. gondii*
199 DNA in marine snails collected from coastal marine water in eastern China. Our data
200 showed an overall prevalence of 1.91% of *T. gondii* DNA in wild marine snails. This
201 result was similar to that reported in oysters (2.61%) (Cong et al., 2017) and mussels
202 (2.48%) (Cong et al., 2019) in China, but was lower than that detected in oysters and
203 mussels from other countries, such as Turkey (Aksoy et al., 2014), Italy (Putignani et
204 al., 2011), USA (Marquis et al., 2015) and Brazil (Ribeiro et al., 2015). The reasons
205 for these differences can be attributed to variations in the examined species, detection
206 method, sample size, sampling season, and environmental conditions, and geographic
207 differences.
208 Our genotyping analysis identified two samples: TgWMS1 from *Glossaulax*
209 *didyma* and TgWMS2 from *Rapana venosa* as the genotype ToxoDB #9 (Table 3). In
210 China, ToxoDB #9 is the main genotype and has been detected in various vertebrate
211 hosts from nearly all provinces of China and in cancer patients (Cong et al., 2015;
212 Wang et al., 2015; Pan et al., 2017; Shwab et al., 2018). Also, our previous study
213 identified ToxoDB #9 in oysters (Cong et al., 2017) and mussels (Cong et al., 2019)
214 in China.

Current evidence indicate that changes in the coastal landscape and natural habitats caused by anthropogenic changes and/or climate changes may have contributed to the contamination of offshore waters with a number of terrestrially derived pathogens (Jones et al., 2008; Shapiro et al., 2010; Simon et al., 2013; VanWormer et al., 2016). In agreement with these studies, the multiple logistic regression identified surface runoff near the sampling site (P = 0.023, OR = 3.665, 95% CI: 1.171-11.475), and residential water discharge near the sampling site (P = 0.013, OR = 4.293, 95%CI: 1.361-12.544) as the most likely factors associated with the presence of *T. gondii* DNA in wild marine snails. These results suggest that some measures should be implemented to reduce the transmission of terrestrial pathogens to marine life.

T. gondii oocysts maintained their infectivity to mice after they were kept in seawater (15 ppt NaCl) at 4^{o}C up to 24 months (Lindsay and Dubey 2009). Therefore, contamination of coastal habitats with *T. gondii* oocysts represents a huge health risk to humans and marine wildlife; infection has been already reported in both offshore and pelagic marine mammals (Shapiro et al., 2015). Therefore, the presence of *T. gondii* DNA contamination in wild marine snails is a serious environmental and public health risk due to the possibility of tranmission of infection via ingestion of raw seafood or seaside recreational activities near urbanized bays (Jones et al., 2008; Jones et al., 2009; Simon et al., 2013; VanWormer et al., 2016).

5. Conclusion

This study detected, for the first time, *T. gondii* DNA in wild marine snails collected from Shandong province, eastern China. Surface runoff and residential water discharge were identified as variables that can increase the risk of the presence of *T. gondii* DNA in wild marine snails. Our findings showed that marine recreational waters in eastern China are contaminated with *T. gondii*, which in turn increases

human risk for infection. These results provide baseline data of *T. gondii* prevalence in marine snails in eastern China. More studies are required to determine the prevalence of *T. gondii* in other coastal regions of China. The presence of *T. gondii* DNA in marine snails is not an accurate proxy for feline fecal pollution in urbanized bays or marine ecosystems and therefore, more studies involving other marine species are warranted.

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Conflict Of Interest Statement

All authors have no conflict of interest to declare.

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Figure legend:

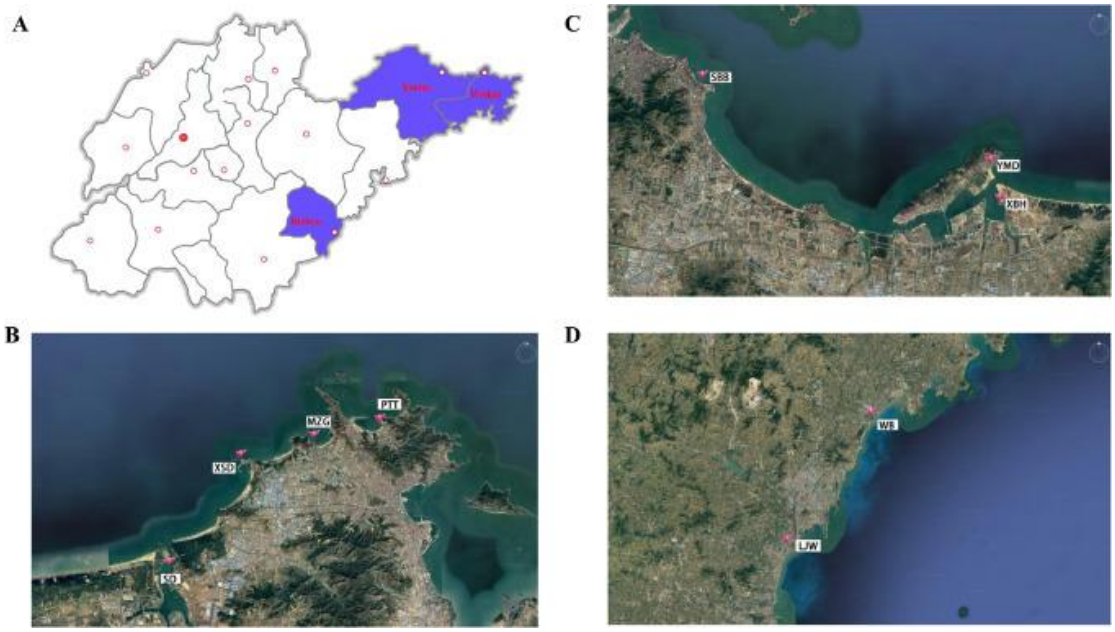

Fig. 1. The study sampling sites in Shandong province, eastern China. (A) Overall sampling cities (Weihai, Yantai and Rizhao). (B-D) Sampling sites of Weihai, Yantai and Rizhao, respectively.

Table 1: Univariate analysis of the risk factors associated with the presence of *T. gondii* DNA in wild marine snails in eastern China

Risk factors	No. tested	No. positive	Prevalence (%)	*P*-value
Species of snail				
Rapana venosa	438	13	2.97	0.79
Monodonta labio	380	0	0	
Glossaulax didyma	388	10	2.58	
Sampling site				
Weihai	436	8	1.83	0.59
Yantai	365	9	2.47	
Rizhao	405	6	1.48	
Sampling time				
Spring	253	9	3.56	0.41
Summer	294	3	1.02	
Autumn	333	6	1.80	
Winter	326	5	1.53	
Surface runoff near sampling site				
Yes	454	18	3.96	0.04
No	752	5	0.66	
Residential water discharge near sampling site				
Yes	219	13	5.94	<0.01
No	987	10	1.01	
Livestock farm near sampling site				
Yes	245	11	4.49	<0.01
No	961	12	1.25	
Total	1,206	23	1.91	

437 **Table 2:** Results of the multiple logistic regression model including parameter
438 estimates and odds ratios.
439

Coefficient	Estimate	Standard error	Odds ratio (95% CI)	*P* value
Intercept	2.932	0.805	–	0.000
Surface runoff nearing the sampling site: Yes	1.227	0.594	3.413 (1.07 – 10.94)	0.039
Residential water discharge near sampling site; Yes	1.384	0.597	3.990 (1.24 – 12.87)	0.021

Table 3. Genetic characterization of *Toxoplasma gondii* amplified DNA detected in wild marine snails in eastern China

Isolate ID	Host	Location	SAG1	5'+3' SAG2	Alternative SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Genotype
GT1	Goat	United States	I	I	I	I	I	I	I	I	I	I	I	Reference, ToxoDB #10
PTG	Sheep	United States	II/III	II	II	II	II	II	II	II	II	II	II	Reference, ToxoDB #1
CTG	Cat	United States	II/III	III	III	III	III	III	III	III	III	III	III	Reference, ToxoDB #2
MAS	Human	France	u-1*	I	II	III	III	III	u-1*	I	I	III	I	Reference, ToxoDB #17
TgCgCa1	Cougar	Canada	I	II	II	III	II	II	II	u-1*	I	u-2*	I	Reference, ToxoDB #66
TgCatBr5	Cat	Brazil	I	III	III	III	III	III	I	I	I	u-1*	I	Reference, ToxoDB #19
TgCatBr64	Cat	Brazil	I	I	u-1	III	III	III	u-1	I	III	III	I	Reference, ToxoDB #111
TgRsCr1	Toucan	Costa Rica	u-1	I	II	III	I	III	u-2	I	I	III	I	Reference, ToxoDB #52
TgWMS1	*Glossaulax didyma*	Shandong	u-1	II	II	III	III	II	II	III	II	II	I	ToxoDB #9
TgWMS2†	*Rapana venosa*	Shandong	nd	II	II	III	III	II	nd	III	II	II	I	ToxoDB #9

* u-1 and u-2 represent unique RFLP genotypes, respectively. † sample was genotyped at 9 loci only.

nd: no data.