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Toxoplasma gondii in mollusks and cold-blooded animals: a systematic review

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Review

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Abstract

Toxoplasma gondii (*T. gondii*) is known for its ability to infect warm-blooded vertebrates. Although *T. gondii* does not appear to parasitize cold-blooded animals, the occurrence of *T. gondii* infection in marine mammals raises concerns that cold-blooded animals (frogs, toad, turtles, crocodiles, snakes, and fish) and shellfish are potential sources of *T. gondii*. Therefore, this systematic review aimed to determine the prevalence of *T. gondii* in mollusks and cold-blooded animals worldwide. We searched PubMed, ScienceDirect, ProQuest, Scopus, and Web of Science from inception to 1 August 2020 for eligible papers in the English language and identified 26 articles that reported the prevalence of *T. gondii* in mollusks and cold-blooded animals. These articles were subsequently reviewed and data extracted using a standard form. In total, 26 studies [involving 9 cross-sectional studies including 2988 samples of cold-blooded animals (129 positive cases for *T. gondii*) and 18 cross-sectional studies entailing 13 447 samples of shellfish (692 positive cases for *T. gondii*)] were included in this study. Although this study showed that shellfish and cold-blooded animals could be potential sources of *T. gondii* for humans and other hosts that feed on them, further investigations are recommended to determine the prevalence of *T. gondii* in shellfish and cold-blooded animals.

Introduction

Toxoplasma gondii (*T. gondii*) is an obligate, intracellular, zoonotic, protozoan parasite that infects nearly all warm-blooded animals including humans, livestock, and some species of marine mammals as intermediate hosts and it is estimated that about 30% of the human population is affected by this parasite. In intermediate hosts, *T. gondii* multiplies by asexual reproduction leading to intracellular cysts in muscles and other organs. However, in felids as final hosts, sexual reproduction leads to discharge oocysts in their feces (Tenter *et al.*, 2000; Dubey *et al.*, 2008; Taghadosi *et al.*, 2010; Aguirre *et al.*, 2019). In immunocompetent humans, *T. gondii* infection is usually asymptomatic but may result in influenza-like symptoms. Although *T. gondii* can be serious, particularly for pregnant women, immunosuppressed individuals, or congenitally infected offspring (Tenter *et al.*, 2000). Among immunocompromised individuals, *T. gondii* infection is common in late acquired immunodeficiency syndrome (AIDS), in which 25% of patients develop toxoplasmic encephalitis (Jones *et al.*, 2001; Aspinall *et al.*, 2002). If *T. gondii* infection occurs during pregnancy, it can cause miscarriage or congenital anomalies that affect the brain, eyes, or other parts of the fetus (Kim *et al.*, 2009). Disseminated toxoplasmosis and mental disorders can occur years after exposure to the parasite, and eye diseases have significant lifelong effects on a person's quality of life (Havelaar *et al.*, 2012). The main routes of transmission of the infection are the contact with soil contaminated with *T. gondii* oocysts excreted from infected felids, ingesting meat containing encysted bradyzoites, consumption of water and vegetables containing sporulated oocysts, passing the parasite from the mother to the fetus through the placenta, blood transfusions, and organ transplants (Dubey and Beattie, 1988; Taghadosi *et al.*, 2010; Koutsoumanis *et al.*, 2018).

The viability of *Toxoplasma* oocysts varies according to environmental conditions. The oocyst can remain infectious in areas with high and low humidity as well as in hot and dry regions (Walker *et al.*, 1992). Sporulated *T. gondii* oocysts can remain infectious in soil for at least 18 months (Esmerini *et al.*, 2010). Freshwater runoff and drainage canals can transport cat feces containing oocysts in the soil to coastal waters through rivers and streams; the oocysts can contaminate freshwater and marine fauna (Fayer *et al.*, 2004). The oocysts can survive for at least 6 months in seawater. In aquatic environments, bivalve shellfish such as mussels, oysters, clams, and cockles are filter feeders that have the unique ability to concentrate and retain suspended particles, including *T. gondii* oocysts from the environment after filtering a large volume of water (Putignani *et al.*, 2011; Coupe *et al.*, 2018). Experimental studies have shown that oocysts may remain viable for up to 85 days in oysters (Lindsay *et al.*, 2004). The specific small subunit ribosomal RNA of *T. gondii* could be detected for up to 21 days after exposure in artificially exposed mussels, but viable oocysts were detected for only 3 days (Arkush *et al.*, 2003). Consumption of waterborne pathogens leads to the storage of these pathogens in bivalves' tissues (Ribeiro *et al.*, 2015). Massie *et al.* showed that *T. gondii* is not capable of infecting invertebrates and the parasite is unable to spread or survive in

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visceral organs such as muscles. Nevertheless, oocysts may be concentrated in organs such as the digestive tract and others that are in close contact with water containing the organism (Massie *et al.*, 2010). Bivalve shellfish are known as excellent beacons for monitoring the health of estuarine ecosystems (Marquis *et al.*, 2019) and can act as paratenic hosts for *T. gondii* oocysts (Cong *et al.*, 2019) that in these hosts, the parasite lives without any developmental stage and is then transmitted to the next host. Bivalve shellfish is a source of infection for marine mammals when consumed as food (Cole *et al.*, 2000). In some areas, bivalves are preyed upon by sea otters and other marine mammals, so obtaining and concentrating *T. gondii* oocysts by bivalves plays an important role in the mechanism of transmission of this parasite from land to sea (Miller *et al.*, 2008). The occurrence of toxoplasmosis in marine mammals raises concerns that cold-blooded marine animals could be potential sources of *T. gondii* infection (Omata *et al.*, 2005). Cole *et al.* in 2000 showed that marine mammals could become infected by consuming invertebrates. Besides, oysters can be a danger to public health because people like to eat oysters in nature. In some countries, mussels are very popular because of their taste and nutritional value. However, they may represent a danger to people given the oysters are eaten raw in most coastal areas of the world, such as China (Cong *et al.*, 2019).

In the case of fish, a study in goldfish (*Carassius auratus*) showed that *T. gondii* tachyzoites could persist for up to 3 days after infection by intramuscular inoculation. This has also been confirmed by polymerase chain reaction (PCR) analysis of inoculated tissues and bioassay in mice with homogeneous tissues of infected fishes by intraperitoneal inoculation (Omata *et al.*, 2005). Given that experimentally the tachyzoites of *T. gondii* survive for more than 7 days in cell culture at 37°C, it does not appear that the infection of the organism in cold-blooded animals is epidemiologically significant under natural conditions (Omata *et al.*, 2005). Because oocysts-carrying fishes may be able to mechanically transmit viable parasites to larger predators at the top level of the food chain, including marine mammals, different routes of transmission should be considered. A new route for oocyst transmission involves the complex interaction of suspended bioparticles, biofilms, small invertebrates, and gastropods. Extracellular polymeric materials are relatively abundant in the marine environment and are involved in the transmission of soil-derived pathogens, including *T. gondii*, by combining marine macroaggregates or adhering to biofilms on the surface of seaweeds or other benthic organisms (Wotton, 2004; Shapiro *et al.*, 2014). Most marine mammals feed on aquatic cold-blooded animals, and *T. gondii* does not multiply in cold-blooded animals (Bossart, 2011). *T. gondii* causes mortality in free-range marine mammals, including some endangered species (Cole *et al.*, 2000; Conrad *et al.*, 2005; Di Guardo and Mazzariol, 2013). Certain marine mammals (e.g. seals) serve as food animals for humans (Forbes *et al.*, 2009; Tryland *et al.*, 2014; Reiling *et al.*, 2019). Therefore, fish can affect public health by infecting marine mammals. Also, the risk of *T. gondii* in fish, acting as carriers of oocysts on the body surface or transiently in the guts, is an occupational issue, and workers, especially pregnant women, should be aware of the risk of exposure to parasite during the primary production stages (fishing and harvesting) and while moving raw fish to produce fishery products and even some fish are eaten raw (Marino *et al.*, 2019). The meat and eggs of reptiles are considered a delicacy as well as their leather has attracted the attention of many consumers and has led to the breeding of species such as crocodiles (Magnino *et al.*, 2009; Hoffman and Cawthorn, 2012). Considering the importance of mollusks (shellfish) and some cold-blooded animals as seafood and the absence of sufficient information on the prevalence of *T. gondii* in mollusks and cold-blooded animals worldwide, this article aims to help to better understand the potential of these animals to transmit toxoplasmosis to humans based on the available literature.

Methods

Study design

This systematic review was performed following the preferred reporting items for Systematic Reviews and Meta-Analysis statements (PRISMA) (Moher *et al.*, 2015). The methods of this systematic review have already been published in the PROSPERO database under the following number CRD42020203071.

Search strategy

We identified original articles on the prevalence of *T. gondii* in mollusks and cold-blooded animals worldwide published in the English language from inception to 1 August 2020. A comprehensive literature search was conducted in PubMed, ScienceDirect, ProQuest, Scopus, and Web of Science using the terms (with the Boolean term OR): 'toxoplasmosis' OR '*Toxoplasma gondii*' OR '*T. gondii*' and cold-blooded and mollusk search terms (with the Boolean term OR): 'reptile' OR 'snake' OR 'fish' OR 'shellfish' OR 'bivalve shellfish' OR 'oyster' OR 'mussel' OR 'mollusk' OR 'snail' OR 'turtle' OR 'cold-blooded'. *Toxoplasma* search terms and cold-blooded and mollusk search terms were combined with the AND Boolean term. Also, the bibliographies of any eligible papers identified were studied for additional references.

Inclusion and exclusion criteria

We imported English language papers that have been formally published as journal articles and studies investigating the prevalence of *T. gondii* in mollusks and cold-blooded animals by microscopic techniques as well as molecular and serological methods. Narrative reviews, systematic reviews, experimental studies, and dissertations were excluded.

Study selection and data extraction

Two independent reviewers assessed titles and abstracts to obtain articles that met the inclusion criteria. If the title and abstract of the article could not be rejected with certainty by both researchers, the full text of the article was retrieved and assessed for eligibility. In this study, researchers did not have any disagreement and the coefficient of agreement (κ) was 100%. In the next step, two investigators extracted the data related to the retrieved papers and placed them in summary tables under the following headings: first author's last name, year of publication, place of study, samples, diagnostic methods, genes, type of antibody, cut-off, the total number of samples, number of *Toxoplasma* positive, and genotypes. The lists of extracted information are presented in Tables 1 and 2.

The quality of publications

In this review, the methodological quality of each of the selected publications was estimated based on the Newcastle-Ottawa Scale criteria (Stang, 2010). This checklist includes three different categories, namely selection, comparability, and exposure. One of these cases is two options and can get two points in the evaluation. The selected publications were then classified as low quality (1–2), moderate quality (3–5), and high quality (6–7). The quality scores of different eligible studies are represented in online Supplementary Table S1.

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Table 1. Data characteristics of the included cross-sectional studies for the prevalence of *T. gondii* in cold-blooded animals

Id	First author's last name (year of publication)	Place of study	Type of animals	Samples	Diagnostic methods	Type of antibody	Cut-off	Genes	Total number of samples	*Toxoplasma* positive *n* (%)
1	Levine and Nye (1976)	USA	Frog and toad	Brain, small intestine, lung, heart, spleen, liver and kidney	Microscopic	–	–	–	183 (Frog: 171 and toad: 12)	1 (0.54) [Frog: 1 (0.58) and toad: 0 (0)]
2	Taghadosi *et al.* (2010)	Iran	Fish (Salmonidae)	Serum	ELISA	IgM and IgG	⩾0.183	–	50	IgM: 5 (10) and IgG: 0 (0)
3	Zhang *et al.* (2014)	China	Fish (*Hypophthalmichthys molitrix*, *Cyprinus carpio*, *Monopterus albus* and *Carassius auratus*)	Digestive tract tissues	PCR	–	–	–	1172 (*Hypophthalmichthys molitrix*: 456, *Cyprinus carpio*: 309, *Monopterus albus*: 98 and *Carassius auratus*: 309)	1 (0.08) [*Hypophthalmichthys molitrix*: 1 (0.22), *Cyprinus carpio*: 0 (0), *Monopterus albus*: 0 (0) and *Carassius auratus*: 0 (0)]
4	Aakool and Abidali (2016)	Iraq	Fish (*Cyprinuscarpi*)	Muscle, liver, enteric and gill	PCR	–	–	B1	96 [Muscle: 24, liver: 24, enteric: 24 and gill: 24]	18 (18.75) [Muscle: 0 (0), liver: 0 (0), enteric: 16 (66.66) and gill: 2 (8.33)]
5	Nasiri *et al.* (2016)	Iran	Reptile (snakes)	Brain	Nested-PCR	–	–	GRA6	68	55 (80.88)
6	Feitosa *et al.* (2017)	Brazil	Reptile (turtle, snakes, lizards and crocodiles)	Serum	MAT	IgM and IgG	⩾25	–	12 (Turtle: 5, snakes: 2, lizards: 2 and crocodiles: 3)	4 (33.3) [Turtle: 2 (40), snakes: 0 (0), lizards: 0 (0) and crocodiles: 2 (66.66)]
7	Anah and Al-Mayali (2018)	Iraq	Reptile (snakes)	Liver, heart, kidney and brain	PCR	–	–	18S rRNA	30	5 (16.66) [Liver: 4 (80), heart, kidney and brain: 1 (20)]
8	Marino *et al.* (2019)	Italy	Fish	Intestines, gills, and skin/ muscles	Real-time PCR and digital PCR (dPCR)	–	–	–	1293 (441 pools; each pool: variable; skin/muscle: 147, gill: 147 and intestine: 147)	32 (7.26) [skin/muscle: 16 (10.88), intestine: 11 (7.48) and gills: 11 (7.48), in one group of *Boops boops*, DNA was detected in all types of tissues]
9	Ferreira *et al.* (2020)	Brazil	Reptile (crocodiles)	Serum	MAT and IHAT	IgM and IgG	MAT: 1:25 and IHAT: 1:8	–	84	MAT: 8 (9.5), IHAT: 26 (30.95) and MAT/IHAT: 7 (8.33)

ELISA, enzyme-linked immunosorbent assay; MAT, modified agglutination test; IHAT, indirect hemagglutination test; PCR, polymerase chain reaction; IgM, immunoglobulin M; IgG, immunoglobulin G; n, number.

Table 2. Data characteristics of the included cross-sectional studies for the prevalence of *T. gondii* in mollusks (shellfish)

Id	First author's last name (year of publication)	Place of study	Samples	Diagnostic methods	Genes	Total number of samples	*Toxoplasma* positive by molecular methods *n* (%)	Genotypes: *n*
1	Miller *et al.* (2008)	USA	Haemocytes and D	TaqMan, conventional PCR and PCR–RFLP	B1 and SAG1	1109	1 (0.09)	X
2	Esmerini *et al.* (2010)	Brazil	Tissues and the enclosed liquid	Nested-PCR and PCR–RFLP	B1	600 [300 (60 pools; each pool: 5 oysters) and 300 (20 pools; each pool: 15 mussels)]	10 (1.66) (2 pools of oysters (3.33) and 0 pools of mussels (0))	–
3	Putignani *et al.* (2011)	Italy	H, G, and D	Nested-PCR and FLAG	B1	1734 (186 pools; each pool: Variable; H: 62, G: 62 and D:62)	2 pools (1.08) [G pool: 1 (1.61), H pool: 1 (1.61) and D pool: 0 (0)]	–
4	Aksoy *et al.* (2014)	Italy	G and D	Real-time PCR and HRM	B1	53	7 (13.2) [D: 4 (7.54), G: 1 (1.88) and DG: 2 (3.77)]	–
5	Zhang *et al.* (2014)	China	Digestive tract tissues	PCR	ITS1	398	0 (0)	–
6	Shapiro *et al.* (2015)	USA	H	Simplex PCR screening and multiplex genotyping assays and PCR-RFLP	ITS1 and B1	959	13 (1.4)	I: 2, II/III: 1, X: 2 and atypical: 2
7	Ribeiro *et al.* (2015)	Brazil	G and D	PCR and nested-PCR	SAG1	624 (208 pools; each pool: 3 shellfish; D: 208 and G: 208)	PCR = 0 (0) and Nested-PCR = 17 (4.08) [D: 4 (1.92) and G: 13 (6.25)]	–
8	Marangi *et al.* (2015)	Italy	–	qPCR and HRM	B1	113 (Turkey = 53 and Italy = 60)	Turkey = 7 (13.2) and Italy = 0 (0)	–
9	Marquis *et al.* (2015)	USA	Rectum, G and mantle tissue	PCR	–	230	4 (1.73)	–
10	Staggs *et al.* (2015)	USA	H, G and D	qPCR or endpoint PCR	Rep 529 gene	41 (Morro Bay: 15 and Point Lobos: 26)	19 (46.34) [Morro Bay = 5 (33.3): H: 4 and G: 1] and [Point Lobos = 14 (54): H: 7, G: 7, and D: 1]	–
11	Cong *et al.* (2017)	China	H, G and D	TaqMan, conventional PCR and PCR–RFLP	B1	998	26 (2.61%)	#9: 1
12	Monteiro *et al.* (2019)	Brazil	G, GIT and IL	Nested-PCR	B1	400 (40 pools; each pool: 10 shellfish; G: 40, GIL: 40 and IL: 40)	7 (5.83) [G: 2 (5), GIT: 3 (7.5) and IL: 2 (5)]	–
13	Coupe *et al.* (2018)	New Zealand	H	Nested-PCR, RT-PCR and PCR–RFLP	dhps	104	13 (12.5)	–
14	Cong *et al.* (2019)	China	H, G and D	Semi-nested PCR	B1	2215	55 (2.48) [D: 23 (1.04), H: 33 (1.49), G: 5 (0.23), DH: 4 (0.18), HG: 1 (0.04), and DG: 1 (0.04)]	#9: 4
15	Marquis *et al.* (2019)	USA	Mantle, G and rectum	qPCR	–	1440	446 (31)	–
16	Tedde *et al.* (2019)	Italy	Internal tissues	Semi-nested PCR	B1	1620 (135 pools; each pool: 12 shellfish)	28 pools (20.74) (each pool: 12 shellfish)	–
17	Silva *et al.* (2020)	Brazil	G and visceral mass	Nested-PCR	SAG1	400 (80 pools; each pool: 5 shellfish)	2 pools (2.5) (each pool: 5 shellfish)	–
18	Santoro *et al.* (2020)	Italy	D	qPCR and semi-nested PCR	B1	409	43 (10.5)	–

H, hemolymph; G, gill; D, digestive gland; GIT, gastrointestinal tracts; IL, intervalvular liquid: DH, digestive gland and hemolymph; HG, hemolymph and gill; DG, digestive gland and gill; PCR, polymerase chain reaction; PCR–RFLP, restriction fragment length polymorphism; qPCR, quantitative PCR; FLAG, real-time PCR fluorescent amplicon generation assay; HRM, high-resolution melting analysis; n, number.

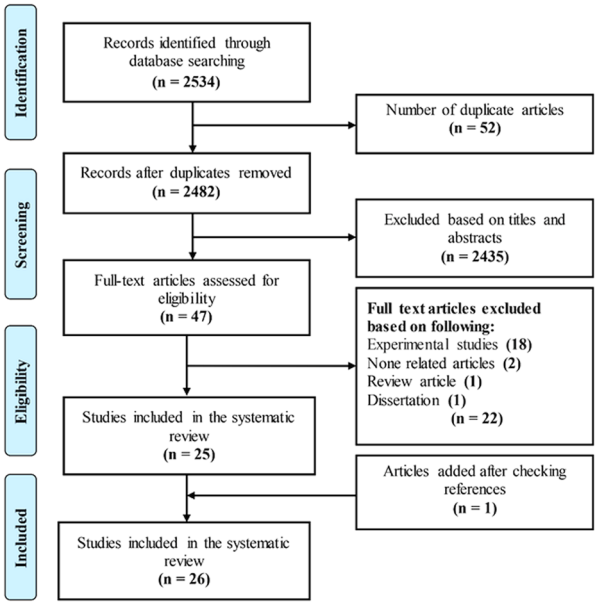

Fig. 1. Flow diagram of the study design process.

Results

Study identification and selection

In the initial literature search, a total of 2534 records were identified; after removing duplicates and non-eligible papers based on title and abstract, 47 articles were selected for full-text screening. Of 47 studies, 18 articles were excluded because they were experimental design, two papers had irrelevant outcomes, one was a review article, and one was a dissertation. In addition, an article was added while checking references. Finally, 26 articles were reviewed. Cross-sectional articles published from 1976 to 2020 were included in the systematic review. Figure 1 briefly shows the search process in this systematic review article.

Prevalence of T. gondii *in cold-blooded animals*

Cold-blooded animals in which the prevalence of *T. gondii* has been studied include frogs and toad (Levine and Nye, 1976), turtles (Feitosa *et al.*, 2017), crocodiles (Feitosa *et al.*, 2017; Ferreira *et al.*, 2020), snakes (Nasiri *et al.*, 2016; Anah and Al-Mayali, 2018), and fish (Taghadosi *et al.*, 2010; Zhang *et al.*, 2014; Aakool and Abidali, 2016; Marino *et al.*, 2019). One of these studies examined crocodiles and turtles (Feitosa *et al.*, 2017). Fish and oysters were also studied in one study (Zhang *et al.*, 2014). The included studies in our systematic review study were conducted in Iran ($n = 3$), followed by Brazil ($n = 2$), USA, Iraq, Italy, and China ($n = 1$).

A total number of 2988 samples of cold-blooded animals in nine studies were entered into the systematic review, including 146 serum samples (50 fish and 96 reptiles) were evaluated for anti-*T. gondii* IgG and IgM antibodies using different serologic tests [enzyme-linked immunosorbent assay (ELISA), modified agglutination test (MAT), and indirect hemagglutination test (IHAT)] out of which 17 cases (5 fish and 12 reptiles) were positive for anti-*T. gondii* IgG and IgM antibodies. In a study by Ferreira *et al.* (2020), the number of positive cases was calculated based on MAT and IHAT findings. The result of the MAT is included here. Also, 2659 samples (1268 fish and 441 pools from 1293 fish as well as 98 reptiles) of different tissues, such as brain, intestines, lung, heart, spleen, liver, kidney, digestive tract tissues, gill, and skin/muscles, were examined for the prevalence of *T. gondii* using different molecular tests (PCR, nested-PCR, real-time PCR, and digital PCR) out of which 111 cases (19 fish and 32 pools from fish as well as 60 reptiles) were positive for *T. gondii*. One study used a microscopic method to diagnose *T. gondii* in amphibian (frog and toad), and of the 183 samples examined, only one was positive (Levine and Nye, 1976). In one study, the fishes were not tested separately, and each species was divided into groups of 3, 5, 6, 8 or 10 units according to the size and weight of the fish. From each fish, intestine, gills, and multiple aliquots of skin–skeletal muscles complex (maximum weight 10 g) were divided separately in asepsis (Marino *et al.*, 2019). The genes used in these studies were B1, GRA6, and 18S rRNA (Aakool and Abidali, 2016; Nasiri *et al.*, 2016; Anah and Al-Mayali, 2018). The characteristics of the included studies are depicted in Table 1.

Prevalence of T. gondii *in mollusks (shellfish)*

The prevalence of *T. gondii* in shellfish was investigated in 18 articles. As illustrated in Table 2, the identified studies were conducted in five countries distributed worldwide, including the USA ($n = 5$), Italy ($n = 5$), Brazil ($n = 4$), China ($n = 3$), and New Zealand ($n = 1$). A total number of 13 447 samples (8069 shellfish and 1327 pools from 5378 shellfish) were examined for the prevalence of *T. gondii* using different molecular tests [TaqMan, PCR, nested-PCR, semi-nested PCR, PCR–restriction fragment length polymorphism (PCR–RFLP), real-time PCR, endpoint PCR, real-time PCR fluorescent amplicon generation assay (FLAG), high-resolution melting analysis (HRM), simplex PCR screening, and multiplex genotyping assays] out of which 692 cases (634 shellfish and 58 pools) were positive for *T. gondii*. In several studies, specimens were used as pooled samples (Esmerini *et al.*, 2010; Putignani *et al.*, 2011; Ribeiro *et al.*, 2015; Monteiro *et al.*, 2019; Tedde *et al.*, 2019; Silva *et al.*, 2020). The pools of several shellfish were formed based on the similarity of morphometric characteristics (Silva *et al.*, 2020). Each pool was analysed as an experimental sample. Then, using a digital scale, the maximum amount of sample for DNA extraction was weighed according to the manufacturer of the kit protocol (Tedde *et al.*, 2019). In these studies, the B1 gene of *T. gondii* as the target sequence was the most common gene for molecular assays (Miller *et al.*, 2008; Esmerini *et al.*, 2010; Putignani *et al.*, 2011; Aksoy *et al.*, 2014; Marangi *et al.*, 2015; Shapiro *et al.*, 2015; Cong *et al.*, 2017, 2019; Monteiro *et al.*, 2019; Tedde *et al.*, 2019; Santoro *et al.*, 2020).

Discussion

In this study, the current knowledge about the prevalence of *T. gondii* in mollusks (shellfish) and cold-blooded animals is systematically described. According to Tables 1 and 2, the highest and lowest prevalence rates of *T. gondii* in cold-blooded animals were related to the studies performed by Nasiri *et al.* as 80.88% (55/68) (Nasiri *et al.*, 2016) and Zhang *et al.* as 0.08% (1/1172) (Zhang *et al.*, 2014). Moreover, the highest and lowest prevalence rates of *T. gondii* in shellfish were observed in studies conducted by Staggs *et al.* as 46.34% (19/41) (Staggs *et al.*, 2015) and Zhang *et al.* as 0% (0/398) (Zhang *et al.*, 2014). Differences in prevalence in various regions are influenced by factors such as species, the size of the sample, method of detecting, the type of environment, and geographical location (Cong *et al.*, 2019). The low levels of basic sanitation in the area and the presence of various species of wild and domestic cats may be predisposing factors to increase the incidence of parasites (Ribeiro *et al.*, 2015). Another important factor is the type of tissue samples. The gills and digestive glands are the places where parasites are most often identified

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(Robertson, 2007; Leal and Franco, 2008). Gills are ideal tissues for the detection of the parasite; oysters use a filter-feeding mechanism and can filter 5 L. Therefore, light particles such as *T. gondii* oocysts are retained in the gills (Ribeiro *et al.*, 2015).

In most of the studies included in this systematic review, molecular methods have been used to investigate the prevalence of *T. gondii* in mollusks and cold-blooded animals (Zhang *et al.*, 2014). In the PCR method, the PCR type, the copy number of the target gene in the sample, and the inhibitors in the tissue samples are the main factors that may affect the detection limit. Various tests used in studies (nested-PCR, semi-nested PCR, real-time PCR, digital PCR, TaqMan, PCR–RFLP, endpoint PCR, FLAG, HRM, simplex PCR screening, and multiplex genotyping assays) and targets (B1, ITS1, GRA6, 18S rRNA, rep 529 gene, dhps, and SAG1) seem to be more sensitive than conventional PCR (Hurtado *et al.*, 2001). Among them, the B1 gene, which has 35 copies in the genome, is the most widely used. The ITS-1, a non-coding spacer region, is used as a target for the development of a PCR method for the differentiation of species among apicomplexans such as *Toxoplasma* (Hurtado *et al.*, 2001; Jauregui *et al.*, 2001). This region is conserved and the ITS-1 sequences showed 100% identity in 20 isolates of *T. gondii* (Homan *et al.*, 1997). The results of a study showed that the detection limit of the ITS-1 PCR assay was 500 fg of *T. gondii* DNA. This amount of DNA is present in 4.5 tachyzoites (Opsteegh *et al.*, 2010). In addition, false-positive amplification occurs frequently using the B1 and ITS-1 primer sets. It is not known whether the dhps gene is single or multiple. In one study, this gene was able to detect 50 oocysts in the hemolymph (Coupe *et al.*, 2018). In comparison to dhps gene, the rep529 gene is purportedly the most sensitive (Su *et al.*, 2010) because there are approximately 200–300 copies of this marker in the *T. gondii* genome (Homan *et al.*, 2000; Costa and Bretagne, 2012).

Due to the technical limitations, the detection of the resistant form of the parasite (oocyst) in environmental samples is a scientific challenge. Although the nested-PCR method is very sensitive to detect *T. gondii*, because the oocyst has a very tough wall, it can interfere with DNA extraction. Therefore, it is difficult to detect oocysts in the environment (Ribeiro *et al.*, 2015). *In vitro* encystation, grinding with glass beads, digestion with proteinase K, and the use of heat shocks are various techniques used to break the oocyst wall, but protocols were not standardized according to temperature and the number of freeze/thaw cycles (Dumètre and Dardé, 2003). The nested PCR method confirms the presence of *T. gondii* DNA, but does not differentiate between unsporulated and sporulated oocysts, the differentiation of these oocysts is important in disease transmission, as only sporulated *T. gondii* oocytes can be infectious (Dubey *et al.*, 1998). To investigate this, a study used RT-PCR of the sporozoite-specific SporoSAG gene and detected *T. gondii* sporozoite mRNA in four of seven mussels (Coupe *et al.*, 2018). Although in this study it is possible to detect SporoSAG mRNA in sporulated but non-viable oocysts, it is not possible to claim with certainty that infectious oocysts were found in these mussels (Coupe *et al.*, 2018). In another study, nested PCR and FLAG were used. The results were consistent. Ease of use and speed, no need for post-reinforcement management for faster analysis, reduced risk of amplitude contamination and quantitative interpretation of results are some of the advantages typical of the real-time PCR technology that FLA demonstrates (Putignani *et al.*, 2011). Some studies use the qPCR method. Despite the development of many qPCR techniques, some of them have limitations in the cost and performance of certain dyes in PCR. For example, TaqMan probes are expensive or dyes, which reduces reproducibility and/or analytical sensitivity (Bustin, 2002; Eischeid, 2011). Dyes such as SyberGreen may inhibit PCR, but EvaGreen is more suitable for routine multiplex qPCR applications due to its less inhibitory effect and lack of dye redistribution (Mao *et al.*, 2007; Li *et al.*, 2010; Eischeid, 2011).

To improve the sensitivity of the assay, it is best to use a hemolymph sample instead of whole tissue homogenates, as the hemolymph is less viscous and probably contains less material that may inhibit DNA amplification by PCR (Shapiro *et al.*, 2015). Some authors report detection limits of 100 oocysts in mussel tissues homogenate (Esmerini *et al.*, 2010), five oocysts in mussel hemolymph (Shapiro *et al.*, 2015) and even a single oocyst in mussel hemolymph (Staggs *et al.*, 2015). One study showed that the diagnosis of *T. gondii* in mussels that were experimentally infected using hemolymph and gill tissue was similar, while the detection of the parasite in the digestive gland was more sensitive in later depuration periods (Arkush *et al.*, 2003). Another diagnostic method is bioassay in mice, in which the amount of material orally administered to each mice and sporulated or viable positive samples is important. In one study, the sensitivity of isolation by bioassay was 10^3 oocytes and the sensitivity of detection by nested-PCR was 10^2 oocytes (Esmerini *et al.*, 2010).

Given that production of shellfish has been considered as an industry worldwide and the consumption of contaminated raw shellfish may represent a considerable health threat, the importance of *T. gondii* in raw or undercooked seafood to serve as a method of parasite transmission should not be ignored. China is the world's largest producer and consumer of shellfish (Cong *et al.*, 2019), and France, Spain, and Italy produce two-thirds of all European mussels (Tedde *et al.*, 2019). France produces >90% of cupped oysters (about 150 000 tons per year) and Spain <5000 tons as well as Italy produces <500 tons per year, which is not enough for internal consumption (Putignani *et al.*, 2011). Oysters are cultured for the consumption of humans (Monteiro *et al.*, 2019). In some parts of the world, such as Brazil, oyster farming is a source of income for families (de Souza Sampaio *et al.*, 2019).

Evaluation of the genotype is an important variable in studying the risk of contamination in cold-blooded animals and potential subsequent infection in humans, because infection with different clonal lineages of *T. gondii* offers very different clinical consequences. Type I strains are highly pathogenic in mice and are associated with acquired ocular disease and diffuse congenital toxoplasmosis. However, the detection of type I strains in cases of chronic infection reactivation in immunocompromised patients or placentas with non-congenital infection suggests that they may also be responsible for asymptomatic infections in healthy patients (Dardé *et al.*, 2007). Type II strains are the most common strains in human diseases. These strains are responsible for many cases of asymptomatic toxoplasmosis in Europe and are also pathogenic for immature fetuses and immunocompromised patients (Dardé *et al.*, 2007). Besides the three main clonal lineages of *T. gondii*, atypical and recombinant strains have been identified (Dardé, 2008). The distribution of *T. gondii* genotypes varies in different geographical areas (Lehmann *et al.*, 2006); in North America and Europe, three distinct lineages (types I–III), in South America, very diverse with few lineages, in China, seven genotypes (Zhang *et al.*, 2014). In America, new genotypes X and A have been identified in sea otters (Sundar *et al.*, 2008). Also, the *T. gondii* genotypes isolated from aquatic animals may be consistent with those isolated from terrestrial animals. Given that there is limited information on *T. gondii* genotypes from oysters, fish, and marine mammals in the world, further studies on *T. gondii* genotypes in aquatic animals are needed to provide stronger evidence for *T. gondii* transmission between aquatic animals and other animals, including humans. However, this risk factor was not specifically investigated

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in most studies; accordingly, this is considered as a basic gap. Also, a useful method for assessing the risk of infection in the consumer is to determine the parasitic load and parasite genotype, and the occurrence of *T. gondii* and the severity of the infection can be affected by the parasite form, dose, genotype, and immune status of the host (Santoro *et al*., 2020). There were several limitations in the present systematic review as follows: (1) a limited number of studies have examined the prevalence of *T. gondii* in mollusks and cold-blooded animals; (2) the use of English articles due to lack of fluency in other languages; (3) the use of various diagnostic methods without equal specificities and sensitivities; and (4) lack of access to published information on the prevalence of *T. gondii* in mollusks and cold-blooded animals from many parts of the world.

Conclusion

Toxoplasma gondii can cause infection ranging from asymptomatic or mild infection in immunocompetent people to miscarriage or serious consequences for the fetus in pregnancy, and fatal encephalitis in AIDS patients. Due to the occurrence of *T. gondii* in shellfish and cold-blooded animals, the consumption of these animals as raw or undercooked in some parts of the world can be a serious public health concern. Therefore, people need to be aware of the risk of acquiring *T. gondii* through eating raw seafood, especially fishes and bivalve mollusks. Finally, many questions remain to be answered in future investigations and it is required to carry out further studies to obtain more accurate details regarding the prevalence of *T. gondii* in mollusks and cold-blooded animals.

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