Identification of *Toxoplasma gondii* B1 Gene in Raw Milk from Sistan and Baluchestan Province Using Nested-PCR

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Abstract:

Introduction: The pathogen of Toxoplasmosis, *Toxoplasma gondii*, is an obligate intracellular protozoan, for which all mammals act as intermediate hosts, however cats are definitive hosts. It is a common zoonotic disease and is transmitted through various routes, including direct contact with infected animals, environmental contamination with *T. gondii* oocysts, contaminated food, unpasteurized milk, and also through the placenta. Toxoplasmosis is one of the leading causes of miscarriage, stillbirth, and congenital infection in humans and animals. Considering the lack of studies conducted on the occurrence of toxoplasmosis in humans and animals in Iran, this research aimed to investigate the genetic prevalence of *T. gondii* in raw milk collected from Sistan and Baluchestan province.

Methods: DNA extraction was performed on 224 milk samples from 64 sheep, 64 goats, 64 cows, and 32 camels, which were collected and transported to the laboratory, and then by identifying the *T. gondii* B1 gene using Nested-PCR method, it was evaluated whether this parasite was present in the samples.

Results: 26 samples (11.6%) were contaminated with *T. gondii* and the prevalence of infection in cows, sheep, goats' and camels' raw milk were 6.3%, 17.2%, 14/1%, and 6.3%, respectively. The highest molecular incidence of infection was identified in sheep milk.

Conclusion: The overall conclusion is that the T. gondii prevalence in samples of raw milk, especially in sheep and goat samples, in the study area is very significant. Consuming raw milk, which contained *T. gondii* DNA, increases the risk of transmitting this parasite to humans, therefore it is necessary to thoroughly boil raw milk or consume pasteurized milk.

Keywords: Nested–PCR, Raw milk contamination, *Toxoplasma gondii*.

1. Introduction

Toxoplasma gondii is the causative agent of toxoplasmosis. Most mammals act as intermediate hosts, but cats are definitive hosts. Environmental contamination with *T. gondii* oocysts causes infection in domestic animals and humans, however, humans are also infected through the consumption of food and unpasteurized milk containing tachyzoites of this parasite (1). The tachyzoite, which is formed in the infectious stage, is transmitted through animal fluids and in the acute stage of infection, it may transfer from the bloodstream to milk (2). Lesions associated with subacute infection caused by tachyzoites can damage the brain, lungs, liver, heart, and eyes, leading to blindness. Lesions in the central nervous system are more severe due to the weaker immune system (3). The major risks of this parasitic infection include congenital transmission and severe fetal malformations, such as hydrocephalus, microcephaly, and miscarriage, and also milder malformations like psychomotor retardation, intellectual disability, and chorioretinitis. Toxoplasmosis is also apparently associated with different psychiatric disorders, e.g., schizophrenia, depression, anxiety, aggression, and suicidal behavior (4).

Milk is one of the livestock products that in terms of nutritional value, is considered equivalent to meat. Humans consume milk from animals as a complete food, both during childhood and adulthood. However, if this animal product is consumed raw, it can transmit various infectious diseases to humans and create some problems for its producers (5). If hygiene is not maintained during milking, transportation, and storage processes, traditional dairy products, including raw milk, may be contaminated with *T. gondii*; cross-contamination is another route to transfer the parasite to those products (6). In milk, Tachyzoites of this parasite were detectable (7, 8), whose existence has been proven in milk specimens of animals such as sheep, goats, cows, and buffalo, and the highest contamination level was observed in sheep and goats milk (9-12).

Since the diagnosis of *T. gondii* based on clinical symptoms is challenging, the main methods for diagnosing *T. gondii* are the cell culture of infected samples in order to isolate this protozoan (which is time-consuming and expensive) and serological tests and detection of antibodies against this parasite, which may involve cross reactions. Due to the disadvantages of the mentioned methods, currently PCR-based methods are often used (12). The B1 gene, characterized by 35 tandem repeats, is commonly used for highly sensitive and specific PCR identification of *T. gondii* (13). When used in Nested-PCR technique, has shown advantages such as high speed, accuracy, high sensitivity, specificity, and safety, and has led to the detection of this parasite in milk from ruminants and traditional dairy products in certain regions of Iran (6, 11, 14). The important issue is that the PCR-based technique cannot differentiate between viability and inactive *T. gondii* parasites, as it only confirms the presence of genetic material (15). Therefore, to definitively prove the viability and infectivity of the parasite, culture methods (in vitro cell culture) or bioassays in mouse models remain essential as gold standard techniques (16).

The lack of research data on the epidemiology of toxoplasmosis in human and animal populations in Iran, particularly in Sistan and Baluchestan province, has created a significant knowledge gap. This information gap, coupled with traditional animal husbandry practices in the region that facilitates close contact between cats (definitive hosts of the parasite) and livestock (intermediate hosts), as well as the common dietary patterns in some communities in the region that lead to the

consumption of unpasteurized dairy products, led to the design and execution of this study on the genetic prevalence of *T. gondii* in raw milk samples from four important animal species in Sistan and Baluchestan province.

2. Materials and Methods

2.1. Sample collection

During 2024 in Sistan and Baluchestan province, raw milk from 64 cattle, 64 goats, 64 sheep, and 32 camels was manually (with gloves) milked from the udders of the animals (which were previously disinfected with alcohol), and a total of 224 random samples were collected. The samples were transported individually in sterile glass containers on ice to the laboratory within 2 to 4 hours.

2.2. DNA extraction

Genomic DNA was extracted using a Pishgam DNA extraction kit (Iran) in accordance with the manufacturer's instructions, and samples were kept at -20°C until the Nested-PCR test.

2.3. Nested-PCR Amplification of the B1 Gene

The reaction mixture (25 µL total volume) for the first round of PCR amplification consisted 12.5 µL 2X PCR Red Master Mix (Pishgam, Iran), 1.5 µL of external Forward primer (10 picomoles), and 1.5 µL of external Reverse primer (10 picomoles; primer sequences listed in Table 1), 2 µL of genomic DNA, and 7.5 µL water nuclease-free. PCR was run on a thermal cycler (MWG, Germany). The cycling conditions were set as follows: initial denaturation 30 sec at 94 °C, then 35 PCR cycles consisting of denaturation at 94 °C for 15 s, annealing at 45 °C for 30 s, and elongation at 72 ° C for 45 s, were performed, as well as final extension for 10 min at 72 °C.

The secondary amplification was conducted with 1 µL of the primary PCR amplicon (DNA template), the second set of primers, and cycling conditions identical to the first round. PCR products were resolved by 2% agarose gel electrophoresis. A bright band of 197 bp in size was formed, confirming the Identification of the *T. gondii* B1 gene. DNA extracted from tachyzoites of the RH strain and only sterile distilled water were the positive control and the negative control, respectively.

Table 1. Primer sequences used in the Nested-PCR reaction.

Target gene	Primer Name	Primer sequence	Reference	Amplicon size
B1 (primary)	Toxo 1 F	5'-TCAAGCAGCGTATTGTCGAG-3'	R. Chiabchalard et al., 2005	-
B1 (primary)	Toxo 1 R	5'-CCGCAGCGACTTCTATCTCT-3'	R. Chiabchalard et al., 2005	-
B1 (secondary)	Toxo 2 F	5'-GGAACTGCATCCGTTCATGAG-3'	R. Chiabchalard et al., 2005	197bp
B1 (secondary)	Toxo 2 R	5'-TCTTTAAAGCGTTCGTGGTC-3'	R. Chiabchalard et al., 2005	197bp

2.4 Statistical analysis of data

The results were analyzed using SPSS statistical software, the Chi-Square test, and differences were considered significant at ($P< 0.05$).

2.5 Ethical considerations

The milk samples used in this research were collected non-invasively during the normal milking process from animals on farms. This procedure caused no pain, suffering, or additional stress to the animals and was performed in full compliance with the standards for milk production. Special permission was obtained from the owner of the animals during sampling.

3. Result

agarose gel electrophoresis results of the Nested-PCR products used for the amplification B1 gene of the *Toxoplasma gondii* in milk samples are shown in Figure 1. It is clear that the *T. gondii* result is positive since a 197 bp band is formed on the gel. A summary of the prevalence incidence of *T. gondii* in samples of milk from different livestock species in Sistan and Baluchestan province is given in Table 2. Of the total 224 Specimens, 26(11.6) tested positive for *T. gondii* B1 gene. The frequency of *T. gondii* contamination in raw milk specimens from cow, sheep, goat, and camel were 6.3%, 17.2%, 14.1%, and 6.3%, respectively, and the highest molecular prevalence of infection was observed in raw sheep milk. Statistical analysis showed that the infection rate among different animals was not significant ($p > 0.05$).

Table 2. the prevalence rate of *Toxoplasma gondii*.

Species	No. milk samples	Positive
Bovine	64	4(6.3%)
Ovine	64	11(17.2%)
Caprine	64	9(14.1%)
Camel	32	2(6.3%)
Total	224	26(11.6)
P-Value	0.172	

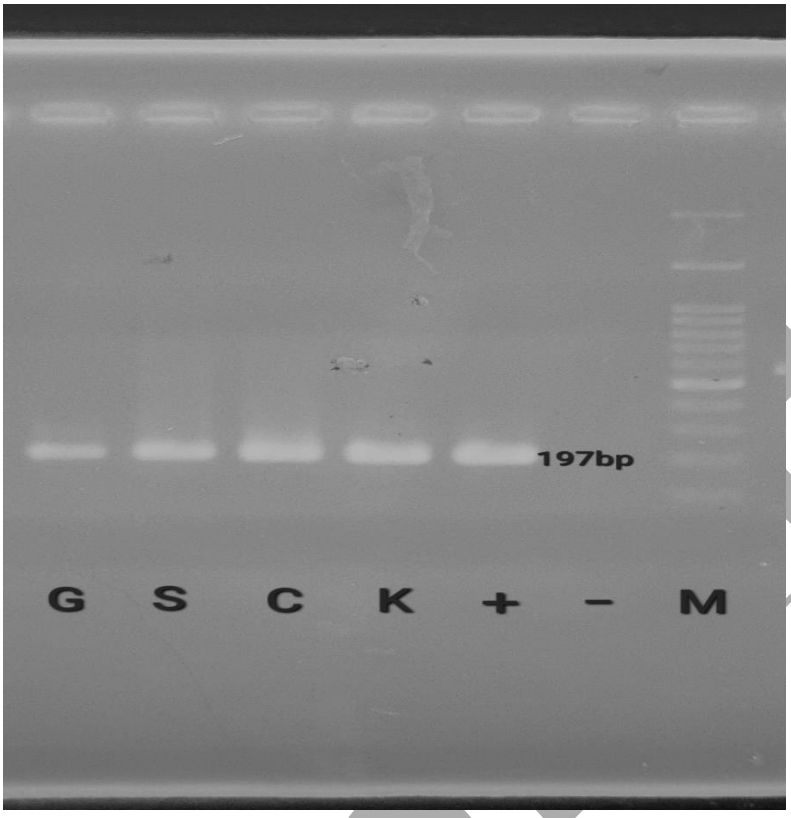

Figure 1. Electrophoresis results of Nested-PCR products related to *T. gondii* B1 gene amplification. "M" means "marker"; the sings of "–" and "+" indicate "negative control" and "positive control" respectively; K = Camel, C = Cow, S = Sheep, and G = Goat are "positive samples".

4. Discussion

The results of the highly sensitive Nested-PCR method for all 224 raw milk samples collected from four livestock species in Sistan and Baluchestan Province showed that *Toxoplasma gondii* was present in 26 (11.6%) of the milk, the highest level of contamination was observed in sheep milk (17.2%). because sheep are likely to be more exposed to environments contaminated with cat feces containing oocysts by free grazing practices, and also some of their physiological features may simplify parasite excretion through milk (2).

A study in Alborz Province similarly detected *T. gondii* in 12% of sheep milk, 10% of goat milk, and 3.5% of cow milk (12). These findings support the present study results and highlight the importance of disease control at both the livestock and dairy product levels.

Another study collected raw milk samples from five livestock species across four seasons in Isfahan, Chaharmahal and Bakhtiari, Khuzestan, and Fars. Provinces. Nested-PCR analysis revealed *T. gondii* in 26 samples sheep milk showed the highest contamination rate (8%), while buffalo milk had the lowest (4.28%) (11). In the current study as well, the highest prevalence was observed in sheep milk samples.

In Fars, Isfahan, and Tehran provinces PCR analysis detected the *T. gondii* B1 gene in 46 (5.17%) milk samples. Fars showed the highest prevalence (18 cases, 6.16%), followed by Tehran (15 cases, 4.76%), and Isfahan (13 cases, 4.6%). Among the positive samples, the greatest rate of *T. gondii* infection was found in goat milk 17 cases (9.44%), followed by sheep 12 cases (6.48%), buffalo 6 cases (3.65), and cow milk 7 cases (3.5%), with the lowest prevalence in camel milk 4 cases (2.5%) (10). In Northwest of Iran was detected *T. gondii* DNA in sheep 16 cases (4.63%) and goat 3 cases (1.07%) milk samples (17). In East Azarbaijan province, detected *T. gondii* in the milk specimens from (13.33%) camels, (3.63%) cows and (3.33%) buffaloes (18). In Assiut, Egypt, detected the *T. gondii* B1 gene in raw sheep milk (10.71%), in raw goat milk (22.73%) by PCR assay (19). Another study in Kayseri Province, Türkiye, A total of 200 specimens of cheese and milk of cow, sheep, goat and water buffalo, detected *T. gondii* in goat (4%) and ewes milk (8%) (20).

In our study, the prevalence of *T. gondii* in sheep milk was reported as 17.2%, while lower prevalences have been reported from studies in Pakistan 14.44%, Brazil 12.04%, and Italy 3.4% (21-23). In our study, the prevalence of *T. gondii* in goat milk was 14.1%, while similar studies from Italy reported a prevalence of *T. gondii* in goat milk of 13%, and lower prevalence in Brazil 6.05%, and higher prevalence from Poland 43%, Pakistan 34.8%, and Thailand 27.9% (21, 24-27). In our study, the prevalence of *T. gondii* in cow milk was reported as 6.3%, while it has been reported as 20% in Pakistan, 76.3% in Serbia, 71% in Brazil, and from Sudan using the ELISA method 13.3% (21, 28-30).

The high prevalence of *T. gondii* infection in sheep may be due to their free-range exposure to infection. These animals are kept in pastures, and environmental contamination with oocysts increases the infection pressure.(31) Epidemiological studies consistently show that cattle and camels exhibit greater intrinsic resistance to Toxoplasma infection than sheep, and their seroprevalence is lower. This resistance appears to be due to a stronger cellular immune response in cattle, which has a better ability to control parasite proliferation (32). However, the findings of studies can be different because each research is influenced by multiple factors, such as geographic location, climate, animal age, grazing systems, feed type, farm hygiene, animal species, laboratory protocols, and milk handling and storage procedures (11).

In raw milk or traditional dairy products (e.g., fresh cheese, yogurt, and cream), *T. gondii* tachyzoites survive for several days at refrigerated temperatures, so the potential risk of transmission is very high, especially for raw or unpasteurized dairy products (1, 7, 33, 34). Since the initial infection probably leads to severe fetal consequences such as microcephaly, hydrocephalus, chorioretinitis, and miscarriage, it vital to pay attention to the threat of transmission for pregnant women, children and immunocompromised individuals (4).

In a systematic review and meta-analysis on the distribution and epidemiological aspects of toxoplasmosis in Iran, the highest prevalence was in sheep at 31%, followed by goats at 27% and cattle at 18% (35). The lack of serological data is a significant gap in the interpretation of our findings. Serological data are crucial for correctly interpreting the presence of the parasite in milk; these data could have helped determine the previous exposure status and differentiate between acute and chronic infection, as parasite shedding in milk is mainly associated with the acute phase.

Unfortunately, due to budget limitations and given that the current study design was cross-sectional, simultaneous access to blood and milk samples was not possible. The reason for using the Nested-PCR technique, as a standard molecular diagnostic tool, in this research for identifying *T. gondii* B1 gene is its high sensitivity and specificity.

The small sample size in camels was mainly due to the challenges of accessing raw milk samples from scattered animals in nomadic and rural areas of Sistan and Baluchestan province. Given that this study is the first to investigate the presence of *T. gondii* DNA in the milk of these animals in this specific region, it is recommended that future studies be conducted with larger sample sizes.

The overall conclusion is that the *T. gondii* prevalence in samples of raw milk, especially in sheep and goat samples, in the study area is very significant. Milk and dairy products are essential parts of the household diet, particularly for children, pregnant women, and lactating mothers, who represent high-risk groups. Therefore, given the zoonosis potential of this parasite and the risk of toxoplasmosis transmission from raw milk, consuming pasteurized milk or thoroughly boiling raw milk is highly recommended. Since cats, the definitive host of this parasite, are commonly found on livestock farms, so to prevent infection, it is essential to minimize their contact with animals and feed.

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Authors' Contribution

Study design: M.M, B.A.H, M.N

Experimental design: M.M

original Article drafting: M.M, M.M

Article review and editing: M.M

Conflict of Interest

The authors have declared no conflicts of interest.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

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Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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