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Original Article

Toxoplasma gondii in Milk of Human and Goat from the Desert Area in Central Iran

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Abstract

Background: *Toxoplasma gondii*, an obligate intracellular protozoan, causes toxoplasmosis. The aim of this study was molecular detection of *T. gondii* in breast and goat milk samples by the molecular method in the central Iran.

Methods: Totally, 300 human' and 200 goats' milk samples were collected randomly from different regions of central Iran in 2018. DNA extraction was performed by the salting-out method. Molecular detection of the parasite was done by nested-PCR using the specific primer pairs. Statistical analysis was performed by SPSS 23 using descriptive and Chi-square tests.

Results: Out of 300 human milk samples, 1 sample (0.3%) was infected with *T. gondii*. Out of 200 samples of goat milk, 11 samples (5.5%) showed infection with *T. gondii*. The frequency of infection in goat's milk samples was 4.36% in the south and west, 1.9% in northern regions, and 2% in eastern regions of the province. The statistical analysis showed no significant difference between toxoplasmosis and different geographical regions in the province.

Conclusion: Because of the popularity of the goat milk and the transfection probability with the milk to humans, it is recommended to boil milk prior to use. Furthermore, case contamination of *T. gondii* in the human milk sample showed one of the important paths for infection transmission, which requires further studies.

Introduction

Toxoplasmosis is the most widespread zoonotic disease worldwide caused by an obligate intracellular protozoan called *Toxoplasma gondii* (1). It is one of the most common parasites in the world, which contaminates 15 to 85% of the human population concerning the geographic location (2). The high prevalence of *T. gondii* was reported from different parts of the world (3). It causes infection in warm-blooded mammals, small ruminants, and birds (4, 5). The definitive hosts of this parasite are cat species, predominantly cats (6) and the important intermediate hosts include birds (7), goats (8), cow (9), pig (10), and sheep (11).

The prevalence of *T. gondii* in cats from different regions of the world is ranged from 1.2% to 89.2% (12).Toxoplasmosis is one of the most important problems not only in medicine, but also in veterinary fields (13). It can also cause economic and reproductive losses due to abortion and may endanger the public health by contaminating the livestock products (14). Among domestic animals, sheep and goats are the main sources of human infection (6) and the parasite transmission chain to sheep and goats has a significant epidemic potential (4).

Transmission of this parasite to humans occurs in vertical transmission, which is from placenta to fetus and in horizontal transmission by cats' oocyst consumption, consumption of tissue cysts in raw or semi-cooked meat and intake of tachyzoites in viscera or non-pasteurized milk of some animals (15).

Naturally, most infected humans are asymptomatic and only a small percentage of infected humans show clinical signs of the disease (16). In pregnant women, if the infection is transmitted to the fetus, variable clinical consequences can be observed from benign forms to drastic and fatal issues (17). Infection with this parasite may lead to abortion, stillbirth, and other neonatal consequences; such as mental retardation, microcephaly, hydrocephalus, jaundice, cortisone (inflammation of the posterior portions of the eye), and neonatal death (18).

Parasite excretion in milk was confirmed in several animal species, such as dogs (19), cats (20), camels (21), and mice (22). Moreover, the statistics show that millions of people eat milk and dairy products every day (1) since milk is a whole food highly valuable in terms of protein, minerals, fats, and vitamins, especially for children and elderlies. Raw goat milk consumption is common in rural areas, especially among poor children because it is digested more easily than cow's milk (18). Moreover, the most important method of feeding infants is breastfeeding since mother's breast milk is a complete diet that promotes the child's mental health, physical development, as well as child-mother emotional relationships (23). Mother's breast milk is rich in immunoglobulin and other immune modulators that are essential for the baby's growth and survival, especially in early life. Excretion of *T. gondii* tachyzoites in the mammary gland is possible by milk secretion (24). This can be explained by a report of toxoplasmosis in an infant whose mother had an initial infection with *T. gondii* (25), given the serious complications of toxoplasmosis in neonates with congenital. Moreover, antiparasitic treatment and early diagnosis of congenital toxoplasmosis can reduce the risk of transmission or consequences.

Lack of information on goat and human milk, as potential sources of *T. gondii* transmission and possibility of consuming unpasteurized milk in the dessert area of the central Iran highlighted the importance of the present study. Therefore, we aimed to determine the presence of *T. gondii* in human and goat milk samples in the central area of Iran for the first time using the PCR-based method.

Materials and Methods

Ethical consideration

This study was approved by the ethics committee of Shahid Sadoughi University of Medical Sciences with the code of IR.SSU.SPH.REC.1397.043. Moreover, written informed consent was taken from all participants before any intervention and completed by the patients. They were assured that their information would be confidential and would be used only for the purposes of the research .

Sampling

From July to October 2018, 300 lactating mothers with children under two years were randomly selected from five different regions (northern, eastern, southern, western and central) of Yazd Province, central Iran. During the same period, 200 goats were randomly selected from four different regions (northern, eastern, southern and western) of this Province. The goats were reared for milk production and grazed in the desert before milking. To collect the goats and mothers' milk samples manually, the nipple was sterilized with 70% alcohol and 10 ml milk was milked directly into sterile 15 ml tube using sterile gloves. The samples were then placed in cold box, transferred to the Laboratory of Research Center for Food Hygiene and Safety, Shahid Sadoughi University of Medical Science, Yazd, Iran and stored at -20 °C for next experiments .

DNA Extraction

DNA was extracted from all human and goat milk samples using the salting out method (26). Briefly, the samples were washed by 1 ml lysis buffer (Tris-HCl, 15 mM, pH 7.6; NaCl, 25 mM, $MgCl_2$, 5 mM; Na2HPO4, 15 mM; EDTA, 2.5 mM; sucrose, 1%). The DNA purification was performed by saturated salt solution and the precipitation was done using cold ethanol. Later, the extracted DNA was solution in 100 µl sterile deionized water and stored at -20 °C until the next use. The quantity of extracted DNA was done using a spectrophotometer, nanodrop (ABI, USA).

Detection and identification

The detection and identification was performed using the specific primer pairs of Toxo1-F: 5'-GGAACTGCATCCGTTCATGAG-3' and Toxo2-R 5'-TCTTTAAAGCGTTCGTGGTC-3' with the amplicon size of 193 bp in the first-round and Toxo4-F: 5'-TGCATAGGTG Toxo2-R 5'-TCTTTAAAGCGTTCGTGGTC-3 for amplification of the 94 bp fragment in the second-round PCR (27, 28). Nested-PCR reaction was carried out in 20 µl volume consisting 100 ng DNA as the template, 1X PCR buffer (Ampliqon, Denmark), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 1 U Taq DNA polymerase, and 0.5 mM each primer (SinaClon, Tehran, Iran). The amplification was done using the thermocycler (ABI, USA) with initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and elongation at 72 ° C for 1 min; the final extension was performed at 72 °C for 5 min for the first round. The second round was done by initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 ° C for 1 min; the final extension was performed at 72 °C for 5 min. The PCR products were analyzed using 3% agarose gel electrophoresis with the 50 bp DNA ladder. In order to negative and positive controls, sterile deionized water and genomic DNA from *T. gondii* gifted from Institute Pasteur, Tehran, Iran was applied. All of the experimental tests were done in triplicate.

Serological test

Serological test was performed for the positive molecular test in humans using

ELISA. The experimental test was done in triplicate.

Statistical analysis

The results were analyzed using SPSS 23.0 software (Chicago, IL, USA). Chi-square test was performed for assessing a significant relationship between geographical areas for the occurrence of parasite in milk samples. The differences were considered significant at a *P*-value of < 0.05.

Results

Theoncentration of DNA extracted from human and goat milk samples was 22.2 ±27 and 80.2 ±87, respectively. The purification of the extracted DNA was calculated using OD 260 /280 that were 1.3± 0.1 and 1.4 ± 0.2 for human and goat milk samples, respectively.

Out of 200 goat milk samples, 11 samples (5.5%) contained *T. gondii*. The percentages of goat milk samples infected with *T. gondii* in the northern, eastern, and southern-western regions of Yazd Province were 9.1%, 18.2%, and 36.4%, respectively. The results of Chi-square test showed no significant relationship between geographical area and infection of goat milk with *T. gondii* (Table 1).

Table 1: Prevalence of *Toxoplasma gondii* in goat milk by geographical area

Area	*Positive (%)*	*Negative (%)*	*Total (%)*
North	1 (9.1)	60 (31.7)	61 (30.5)
South	4 (36.4)	35 (18.5)	39 (19.5)
East	2 (18.2)	43 (22.8)	45 (22.5)
West	4 (36.4)	51 (27)	55 (27.5)
Total	11 (100)	189 (100)	200 (100)

P value= 0.27

The molecular detection and identification showed that out of 300 human samples, one (0.3%) had *T. gondii*. The milk sample was from a 37 years old lactating woman. Then, the first serological test was done using ELISA, which was performed on 21 September 2019, showed 1.2 IU/mL IgG and 4.1 IU/mL IgM (more than 11 IU/mL was positive for toxoplasmosis). The repeated serological test was done on 1 February 2020 using ELISA with the results of 2.36 IU/mL IgG and 1.78 IU/mL IgM (more than 11 IU/mL is positive for toxoplasmosis). Therefore, the blood sample was obtained in 8 February 2020. The sample was divided into two separate sterile tubes. One tube was stored at -20 °C and the other was used for DNA extraction. Nested PCR showed a 132 bp fragment using 3% agarose gel electrophoresis alongside the 50 bp DNA ladder. To check the immune system function in our case, an ELISA test was done for assessing IgG, IgM, IgA, and IgE. The results showed 394 mg/dL IgA (reference range: 70-400 mg/dL), 168 mg/dL IgM (reference range: 40-230 mg/dL), 1256 mg/dL IgG (reference range: 700-1600 mg/dL), and 16 IU/mL IgE (reference range: up to 160 IU/mL), which were all normal.

Discussion

People, especially in rural areas of the world are accustomed to drink raw goat's milk and consume freshly produced cheese from raw and unpasteurized goat's milk, considering its favorable taste. This can be a risk factor in the transmission of *T. gondii* parasites that threatens the public health. It is also worth

noting that the rural climate supports survival of oocysts and includes a high prevalence of *T. gondii*. Furthermore, breast milk is the main and vital food for baby growth early in life. Considering the transmission of *T. gondii* to the fetus during pregnancy, it is highly important to determine its transmission through milk.

In this study, we found that 5.5% of the goat milk samples and 0.3% of the human milk samples were infected with *T. gondii*. Besides, goat's geographical area had no significant relationship with milk infection with *T. gondii* parasite. Various studies have investigated this issue. A study (29) from Multan Punjab Pakistan showed that 52% of *T. gondii* parasitic infections was found in goat's blood samples that its prevalence is more than the one in our study. It may be due to the type of specimen taken from goats. In Italy, 13% of the blood and milk samples of a goat population was infected with *T. gondii* (30). In a Brazilian study, goat milk contamination was reported about 6.05%, which is consistent with our results (31). Compared to our results, a lower contamination rate (2.06%) was reported in goat's milk and whey samples using PCR (32) in Brazil. However, in Poland, 65% of the goat milk samples (N = 60) was infected with *T. gondii* (33). Furthermore, in Egypt, 90%, 60%, and 3.33% of goat, sheep, and camel samples had *T. gondii* antibodies, respectively (34). The researchers concluded that raw milk was contaminated by parasitic tachyzoites and could be a source of human infection. They also suggested that limited health programs should be implemented in animal breeding farms to reduce the risk of milk contamination (34). In Mongolia, *T. gondii* DNA was reported to be zero in goat milk, while existence of parasites was observed in sheep and camel milk (35). In Benin, West Africa, 53% of goat blood samples and 1.4% of sheep blood samples had *T. gondii* antibodies (36). The difference in the rates of infection recorded in sheep and goats was determined based on the ambient moisture (36). In northwestern Iran, 4.63% of sheep milk samples and 1.07% of goat milk samples were infected. The lower infection rate in goats than sheep can be attributed to their differences in susceptibility to *T. gondii* and the animals' nutritional habits. Furthermore, presence of *T. gondii* DNA, detected by molecular analysis, increases the transmission odds of this parasite by consuming raw milk and its non-pasteurized products. This is a worrying finding for the public health (4). In this regard, Gazzonis et al. (37) reported that *T. gondii* DNA was found in 20.6% of the goat milk samples and showed that the presence of parasites in goat milk was a risk factor for public health. Another study investigated the presence of *T. gondii* in goat, sheep, buffalo, cattle, and camel milk samples by cell culture, cat biopsy, ELISA, and PCR, respectively. The results showed that goats (10%), summer (76.47%), and Fars province (6.84%) had the highest prevalence rates; whereas, camel (3.12%) and winter (3.92%) had the lowest prevalence rates (1). Higher prevalence was recorded (18) where the prevalence of *T. gondii* in sheep and goat milk samples was 10.48% by microscopic examination by latex agglutination test, and 16% by PCR. Discrepancies in the results with the present study can be due to the difference in applied diagnostic methods, study sites, breeding conditions, and farm management and can be attributed to the moisture of the Egyptian region and thus to the survival of the parasitic oocyst. The high prevalence of *T. gondii* infection indicates continued exposure of sheep and goats to oocysts shed by stray cats, since contamination is severe in fields with poor management conditions (18).

The results of our study also showed that the frequency of 0.3% for *T. gondii* in human milk. Breast milk is the main and vital food for baby growth early in life. Considering the transmission of *T. gondii* to the fetus during pregnancy, it is highly important to determine

its transmission through milk. The molecular detection of *T. gondii* DNA in milk and proving the transmission by this way may make worrying finding for the public health (38). In Egypt, positive specific IgG-IFAT in the serum of 16 rural and 6 urban women as well as in the milk of 10 rural and 2 urban women was found (39). However, parasite infection had no significant association with the serum and milk. Furthermore, a study (40) concluded that the relatively low level of *T. gondii* antibody in serum can be excreted in milk, which may have protective effects on infants. In the evidence, there is a case report from a two-month-old infant with acute toxoplasmosis that acquired the disease during breastfed from his mother. In the mentioned case, the serology test was negative in newborn and the mother was susceptible to the disease agent (40). It seems that the verification of the transmission of *T. gondii* by breastfeeding in humans needs more studies. For approving this phenomenon, other possible routes of toxoplasmosis transmission, such as the consumption of meat and water contaminated with toxoplasmosis agent or transmission from the placenta should be excluded. Among the mentioned routes, the transmission of *T. gondii* oocyte is the most common way by breastfeeding mothers, resulting in acute toxoplasmosis.

Some studies have reported different methods such as PCR, ELISA, fluorescent to detect *T. gondii* (41-43). The evidence showed that PCR-based methods are more sensitive and specific in disease diagnosis compared with serological ones (44). In this study, we used nested PCR, which is a valid method for detecting this parasite with acceptable sensitivity and specificity.

Conclusion

The results approved the presence of *T. gondii* in goat milk. Considering the favorable taste and popularity of this milk, it should be boiled before use. Furthermore, in this study, we introduced a case of toxoplasmosis with a positive PCR-based method and a negative serologic test. This made us more attention to the technical diagnosis of toxoplasmosis, especially in lactating women. This finding needs further investigations due to prove the way of transfer.

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Conflict of interest

The authors declare no conflict of interest.

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