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Molecular detection of *toxoplasma gondii* DNA in goats (*Capra hircus*), sheep (*Ovis aries*), and donkey (*Equus asinus*) milk using PCR in East Azerbaijan province, Iran

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ABSTRACT

Toxoplasmosis, a zoonotic infection that is significant for public health (immunocompromised patients, pregnant women) and veterinary medicine (economic losses in the herd), is caused by an intracellular protozoan parasite belonging to the phylum Apicomplexa called *Toxoplasma gondii*. Consumption of unpasteurized milk and contaminated undercooked meat is a significant source for humans. The present study aimed to determine *Toxoplasma gondii* DNA in sheep, goats and donkeys Milk kept in East Azerbaijan province using the PCR method based on the B1 gene. For this purpose, 100 milk samples, including 45 sheep, 45 goats and 10 donkeys, were collected from different regions of northwestern Iran using direct milking and then transferred to the Food and Aquatic Health Laboratory under refrigerated conditions. The results showed that out of 100 milk samples examined, 16 samples (16%) were contaminated, and *Toxoplasma gondii* DNA was detected in 5 (11.11%) sheep, 9 (20%) goats and 2 (20%) donkeys milk specimen, respectively. These findings indicated that *Toxoplasma gondii* contaminated the raw milk, a human infection source.

1. Introduction

In recent decades, the consumption of donkey milk by humans has been increasing because its compositions are the most similar to woman's Milk due to comparatively poor in fat and protein but rich in lactose and can be consumed as a reliable substitute for babies, children and adults with IgE-mediated cow milk allergy (Vincenzetti et al., 2014). It also modulates the immune response of the intestinal mucosa and is appropriate for older adults. Based on some ethnic beliefs, donkey milk is also suggested for tumor therapy or treatment of conjunctivitis, in aiding the atherosclerosis prevention, and as strong inhibitory activity against some bacteria because of the high contents of lactoferrin and lysozyme (Boughattas, 2017). As well as, goat milk with a casein structure is similar to human milk and can be a good alternative for people and children with allergies to cow's milk. Sheep milk has a high extent of butterfat, but it contains lower saturated fat than goat. It has higher zinc, calcium, and vitamins A, D, and E values (Park et al., 2007; Raynal-Ljutovac et al., 2008).

Consumption of Milk contaminated by tachyzoites or speculated oocysts with water sources, fruits, vegetables, or tissue cysts from meat contaminated with *T. gondii*, an obligate intracellular protozoan, by mammals including humans as intermediate hosts causing toxoplasmosis (Boughattas, 2017). The clinical signs of toxoplasmosis in goats and sheep include neonatal death, abortion, fetal death, and mummification, causing severe economic damages in their industry (Dubey et al., 2020a, 2020b; Sharif et al., 2015) and in humans, ranging from stillbirth, abortion, and other congenital infections to eye disease in acute cases, while in chronic infection the fetal encephalitis is the most prevalent symptom (Dalimi and Abdoli, 2012; Smith et al., 2021; Weiss and Dubey, 2009). *T. gondii* has been found in the Milk of many hosts, such as sheep (da Silva et al., 2015; Luptakova et al., 2015; Rocha et al., 2015; Saad et al., 2018; Sadek et al., 2015; Tavassoli et al., 2013), goats (Amairia et al., 2016; da Silva et al., 2015; Dubey et al., 2014; Khamsian et al., 2021; Mancianti et al., 2013; Saad et al., 2018; Sadek et al., 2015; Tavassoli et al., 2013) and donkeys (Dubey et al., 2020c; Haridy et al., 2010; Mancianti et al., 2014; Martini et al., 2014).

Given that animal milk, especially lately donkey milk, has been gradually rediscovered as a valuable source of nutritious food for people (Polidori and Vincenzetti, 2013), knowledge about hygiene, safety, and microbiological of this. Other milk can be crucially important to some

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consumers (elderly, infants with cow milk allergy when breastfeeding is impossible, cases of low immune system defenses, and healing). So, the present research aimed to determine *T. gondii* DNA in sheep, goats and donkeys Milk kept in East Azerbaijan province using the PCR method according to the B1 gene.

2. Materials and methods

2.1. Sampling

One hundred animals, including 45 goats (*Capra hircus*), 45 sheep (*Ovis aries*), and 10 donkeys (*Equus asinus*) Milk were indiscriminately chosen from different areas of East Azerbaijan province of Iran, From April to November 2019. Milk specimen (200 ml each animal) were collected manually by milking the teats previously disinfected with iodine alcohol and using gloves. The specimen were maintained under refrigeration and transferred to the laboratory of Parasitology, Faculty of Veterinary Medicine, University of Tabriz in sterile micro-tubes, for PCR assay.

2.2. DNA extraction

Fifty ml milk specimen was centrifuged at 2500*g* for 5 min for each investigated animal (Murphy et al., 2002). To avoid interference by casein, one ml of the sediment was re-suspended in 200 μl TE (1 mM EDTA, 10 mM Tris-HCl (pH = 7.6)) and 300 μl 0.5 M EDTA (pH = 8), and centrifuged at 3000*g* for 10 min (Psifidi et al., 2010). After that, the milk pellet was diluted in 200 μl of PBS. DNA was extracted by applying a DNA extraction kit (Cinna Gen, Tehran, Iran) based on the manufacturer's instructions, and the DNA qualities were checked by electrophoresis on the 1% agarose gel.

2.3. PCR amplification

Sensitive and previously reported species-specific primers utilized to amplify a fragment of 529 bp performed the amplification of the B1 gene of *T. gondii* (Homan et al., 2000; Tavassoli et al., 2013). The primer arrangement was TOX4 (CGCTGCAGGGA GGAAGACGAAAGTTG)/ TOX5 (CGCTGCAGACACAGTGCATCTGGATT). PCR buffer, 2 mM MgCl2, 250 μM of each of the four deoxynucleotide triphosphates, 1.25 U Taq DNA polymerase (Fermentas, Germany), 50 pmol of each primer and 5 μL of the extracted DNA. The positive control for *T. gondii* was kindly provided by Dr. Ehsan Ahmadpour from Tabriz University of Medical Sciences, Iran. Sterile water was used as a negative control. The cycling situation was 94 °C for 7 min, followed by 33 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, with a final step at 72 °C for 10 min. Five microliters of each DNA specimen were utilized as the template. PCR products were analyzed by 2% agarose gel electrophoresis followed by DNA-safe stain (Yekta Tajhiz Azma, Iran; Cat no: YT0001) staining. The gel was photographed under a Gel Documentation system (Axygen® Gel Documentation systems, German).

3. Results

Out of 100 milk samples from different animals, 16 samples (16%) were contaminated with *T. gondii*. The results indicated that out of 45 specimens of sheep's Milk, 5 samples (11.11%), out of 45 specimens of goat's Milk, 9 samples (20%) and out of 10 samples of donkey's Milk, 2 samples (20%) were infected with *T. gondii* (Fig. 1). Statistical analysis showed that the infection rate among different animals was not significant ($p < 0.05$).

4. Discussion

T. gondii is one of the worldwide food-borne zoonotic protozoa, infecting humans, ruminants, and other warm-blooded animal species

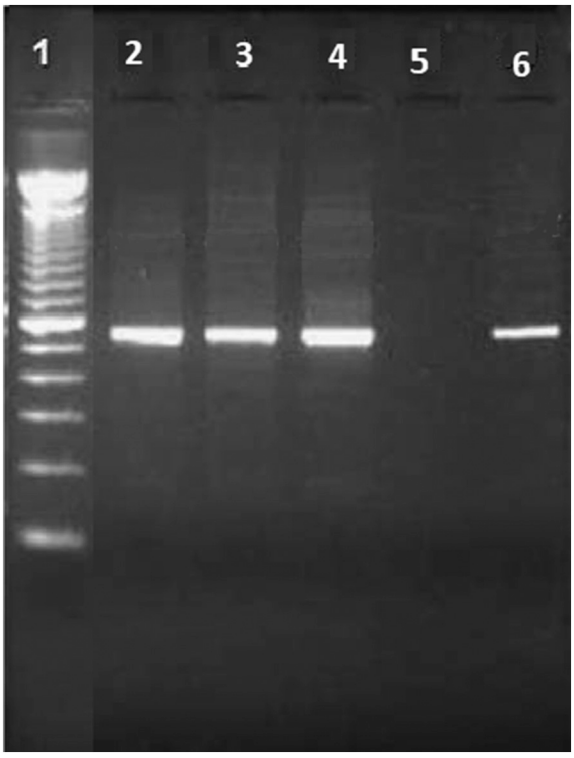

Fig. 1. PCR-Amplified Products Using *T. gondii* Specific Primers: Lane 1, 100 bp Ladder (Fermentas, Germany); lane 2, sheep milk; lane 3, goat milk; lane 4, donkey milk; lane 5, negative control, lanes 6, positive control.

(Sharif et al., 2015). Humans become infected by ingesting food contaminated with oocysts, tachyzoites, or tissue cysts. Using raw Milk from contaminated animals is a risk factor for being infected with toxoplasmosis in humans (Daryani et al., 2014).

The results showed that out of 100 milk samples of animals, *T. gondii* DNA was found in 16 samples (16%). The prevalence of *T. gondii* in goat, sheep and donkey milk was 20%, 11.11% and 20%, respectively.

Various studies have been conducted on this subject with different levels of contamination. For example; Abdel-Rahman et al. (2012) found that 58.90% of the examined goat's milk samples in Egypt had *T. gondii* antibodies. Saad et al. (2018) determined *T. gondii* IgG antibodies in 90% of goat milk specimens in Egypt using ELISA and 3.7% Quantitative polymerase chain reaction (qPCR) (Saad et al., 2018). In Italy, 13% of the goat population's milk and blood specimens were infected with *T. gondii* (Mancianti et al., 2013). In Poland, among investigated 60 milk samples, 65% were positive in Real-time PCR and 43% in nested PCR (Sroka et al., 2017).

In a study conducted by Bezerra et al. (2015), it was revealed that the contamination rate with *T. gondii* in the milk goat was 22.58% (Bezerra et al., 2015). In an Egyptian investigation, about 22.73%goat milk contamination was reported, which agrees with our findings (Sadek et al., 2015). In Brazil, the contamination rate of goat milk specimens with *T. gondii* was reported at 15.78% using the Indirect fluorescent antibody test (IFAT) and 2.06% by PCR (da Silva et al., 2015). Khamsian et al. (2021) stated that out of 200 samples of goat milk, 11 samples (5.5%) were infected with *T. gondii* (Khamsian et al., 2021). In a study by Tavassoli et al. (2013), PCR evaluated 280 milk samples of goats and 345 milk samples of sheep, and the findings revealed that 3 (1.07%) goats and 16 (4.63%) sheep milk specimens were contaminated (Tavassoli et al., 2013). This discrepancy could be explained by the diagnostic methods, environmental conditions, and the presence of cats in the herds (Amairia et al., 2016). *T. gondii* DNA was found in the milk samples of 6.5% sheep from South Bahia (Rocha et al., 2015) and 28% sheep from the Slovak Republic (Luptakova et al., 2015). Disagreements

in the findings of this investigation can be the result of the difference in study sites, farm management, breeding conditions, and applied diagnostic methods and can be due to the moisture of the various regions and, therefore, to the parasitic oocyst survival (Masala et al., 2003).

Only three researches have concentrated on *T. gondii* prevalence in the donkeys' milk matrix.

Haridy et al. (2010) described an infection rate of 46.3% by detecting antibodies against toxoplasmosis in the milk of a pregnant Egyptian donkey female by ELISA. In Europe, Mancianti et al. (2014) studied the *T. gondii* detection in Italian donkeys by molecular tools. Three of the six examined milk specimens were infected by the n-PCR technique. Another Italian investigation concentrating on the safety and quality of Milk in donkeys indicated 22.22% of toxoplasmic parasitemia, which was nearly consistent with our studies (Martini et al., 2014).

The obtained results concluded that there is a high incidence of toxoplasmosis among sheep, goat and donkey milk samples in East Azerbaijan province, Iran. The existence of *T. gondii* DNA in the sheep, goats and donkeys' Milk increases the probability of parasite transmission to humans through raw milk consumption. From the result achieved, boiling or pasteurization of Milk before human consumption is suggested to eliminate the risk of parasite transmission to milk consumers. Moreover, further investigations are necessary to detect the incidence of *T. gondii* DNA in other milk-producing animals and apply influential strategies to control toxoplasmosis.

Declaration of Competing Interest

The authors declare no conflicts of interest relevant to this study.

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