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Molecular prevalence of *Toxoplasma gondii* in sheep from Isfahan, Iran as detected in heart samples by loop-mediated isothermal amplification (LAMP)

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ABSTRACT. Toxoplasmosis is considered an important zoonosis that affects not only humans but also livestock worldwide. Small ruminants are important intermediate hosts of *T. gondii*. This study aimed to determine the molecular prevalence of *T. gondii* in the slaughtered sheep by loop-mediated isothermal amplification (LAMP) assay in Isfahan, Iran. A total of 250 sheep heart samples were collected from five different slaughterhouses in Isfahan, Iran, from October 2017 to May 2018. The LAMP assay was optimized targeting the sequence of 529 bp DNA fragments of *T. gondii* and detected *T. gondii* DNA in 96.4% (241/250) of samples. A significantly higher prevalence of *T. gondii* was recorded among female animals as compared to male animals ($p \leq 0.05$). No statistically significant difference in *T. gondii* prevalence was noted between the seasons, type of animals, and age groups ($p > 0.05$). To the best of our knowledge, the current study is the first to use the LAMP assay to determine the prevalence of *T. gondii* in sheep in Iran. The prevalence obtained in this study by the LAMP assay was higher than the prevalence reported by previous studies that used other methods. This study suggests that sheep meat may play a more prominent role as a source of infection of humans and the LAMP technique can be a recommended tool for accurate diagnosis of toxoplasmosis.

Keywords: *Toxoplasma gondii*, sheep, Loop-mediated isothermal amplification (LAMP), Iran

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INTRODUCTION

Toxoplasma gondii is a zoonotic parasite with worldwide distribution; it is capable of developing in a wide variety of vertebrate hosts (carnivorous and herbivorous mammals and birds), but its final host is the domestic cat and some other members of the Felidae family. While the ingestion of cyst-containing meat may be a common route of infection among carnivores, herbivorous animals must acquire the infection by ingestion of oocysts. Transmission through the placenta usually occurs in the course of an acute but inapparent or undiagnosed maternal infection. In some domestic animals, chronic toxoplasmosis may lead to abortion, stillbirth or congenital defects of the newborn. Diagnosis is important for the control of toxoplasmosis (Dubey, 2009) and several tests are available to confirm the diagnosis. Sabin-Feldman dye test has proven to be reliable and accurate in a range of hosts and is the preferred test in most circumstances. The presence of antibodies against *T. gondii* in the blood is the usual sign for suspecting toxoplasmosis (Tavassoli et al., 2013). In PCR-based techniques, amplification of a specific part of the genome can generate many same copies of the target DNA sequence. Nested PCR (n-PCR) is applied not only to detect a few template copies of pathogens but also to increase the specificity of DNA products (Rostami et al., 2018). Various multi-copy targeting genes including 35-repeat B1 gene, 18S rRNA-, P30-, and 529-bp repeat fragment have been amplified for the detection of *T. gondii* in diverse samples (Rostami et al., 2018). Loop-mediated isothermal amplification (LAMP) is a rapid and efficient molecular assay, with higher specificity and sensitivity than other PCR-based methods (Sun et al., 2017). These characteristic features have stimulated many researchers to use this technique to detect different pathogens including *T. gondii* (Rostami et al., 2018). A systematic literature review/meta-analysis on the status of toxoplasmosis in Iran revealed that the prevalence of the infection is between 33-46% for humans, 26-35% for sheep, 14-42% for goats, 10-28% for cattle, and 22-46% for cats by different detection methods (Foroutan et al., 2018). A study has recently determined the prevalence of *T. gondii* in the Iranian house sparrow (*Passer domesticus*) by LAMP method (Abdoli et al., 2016). However, according to a survey of the literature, there are no data on the prevalence of *T. gondii* in small ruminants obtained with the use of LAMP assay in Iran. Therefore, the current study aimed to determine the molecular prevalence of *T. gondii* in slaughtered sheep as detected by LAMP assay in Isfahan, Iran.

MATERIALS AND METHODS

Area of study

The study was carried out on sheep in Isfahan province (longitude 49° 36'55" 31' E, latitude 30° 43'-34° 27' N), central Iran. The province is located almost in the center of Iran covering an approximate area of 107,027 km². The province has temperate and dry weather, on the whole, and the temperature varies between 10.6°C and 40.6°C on a warm day in the summer season. The mean annual temperature and rainfall have been recorded as 16.7°C, and 116.9 mm, respectively. According to statistics released by the Iranian Ministry of Jahad-e-Agricultural (animal production department) in 2017, in Isfahan province, there are about 1,398,000 sheep and 494,000 goats that produce more than 45.34 million kilograms of meat per year.

Sampling

A total of 250 sheep heart samples were randomly selected from five different slaughterhouses of Isfahan, Iran, from October 2017 to May 2018. The minimum sample size was calculated based on an estimated prevalence of 20%, an error of 5%, and a significance level of 5%, which was found to be 245 samples. However, the sample size was increased to 250 samples. Sampling was performed weekly for 25 weeks and the variables of age and sex were recorded for each animal. Each slaughterhouse was visited 25 times, and heart samples were randomly collected from two sheep for each sampling date. One gram (Approximately 1 cm³) heart sample (free of fat and connective tissue) was cut by a new sterile scalpel blade for every sample (to prevent cross-contamination) and put into sterile labeled sampling bags (75 x 125 mm). The samples were kept in a cold box immediately after collection and were transported to the research laboratory on the same day of the collection under cold conditions (2-4°C). At the laboratory, samples were frozen at liquid nitrogen and crushed in 1.5-mL plastic tubes before lysis. Proteinase K and lysis buffer (50 mL of Tris-HCl, 1 mM of EDTA, pH = 8.0; pH = 7.6, and 1% of Tween 20) were added to the 50mg homogenized heart sample and incubated at 50°C for 24 hours. DNA was isolated from heart samples using a Quick Genomic DNA Isolation Kit (MBST, Iran) according to the manufacturer's guidelines. Extracted DNA was dissolved in 100 µl of double-distilled water and stored at -20 °C until subsequent LAMP assays.

PCR assay

All primer sequences and the positions of the used primers are displayed in Table 1 and Figure 1, respectively (Lin et al., 2012; Zhang et al., 2009). In the beginning, the specificity of the outer LAMP primer pair (F3 and B3; Table 1) was tested using the PCR. The first PCR was performed in 25 μ L total volume containing 1 μ L extracted DNA (DNA extracted from mouse brain infected with *T. gondii* was used as the positive control), PCR buffer (one time), 0.5 U Taq Polymerase (Cinnagen, Iran) 200 μ M of each dGTP, dATP, dCTP, and dTTP (Cinnagen, Iran), 1 μ L of each primer (F3/ B3, 20 μ M, Cinnagen), and 1.5 mM MgCl₂ in automated thermocycler (T100 Thermal

Cycler, Bio-Rad) using the following program: incubation at 95 °C for 4 min to denature double-strand DNA (Initial denaturation step), 35 cycles at 94 °C for 45 s (denaturation step), 55 °C for 45 s (annealing step) and 72 °C for 1.5 min (extension step). The PCR products were visualized under UV light after running on a 1.5% agarose gel and stained with ethidium bromide (1 μ g/mL). To determine the least possible amount of template DNA in a reaction which can be detected by PCR assay, a serial dilution of DNA (positive control) was used and the minimum concentration of the genomic DNA sample (1:25) was selected and used to optimize the LAMP assay (Figure 2).

Table 1. Nucleotide sequences of LAMP primers designed in this study.

Target	Primer name	Nucleotide sequence	Amplon	Reference or source
529-bp repetitive element	F3	5'-CCACAGAAGGGACAGAAGTC-3'	202 bp	(Zhang et al., 2009; Lin et al., 2012)
	B3	5'-TCCGGTGTCTCTTTTCCAC-3'		
	FIP	5'-TCCTCACCCCTCGCCTCATCTAGGACTACAGACGCGATGC-3'		
	BIP	5'-TGGTTGGGAAGCGACGAGAGTCCAGGAAAAGCAGCCAAG-3'		
	LF	5'-TCCAAGACGGCTGGAGGAG-3'		
	LB	5'-CGGAGAGGGAGAAGATGTTCC-3'		



Figure 1. Position of six primers used for the LAMP assay targeting eight conserved regions within the sequence of 529 bp DNA fragment of *T. gondii* (GenBank accession number AF146527). The outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), backward inner primer (BIP), loop forward (LF) and backward (LB)

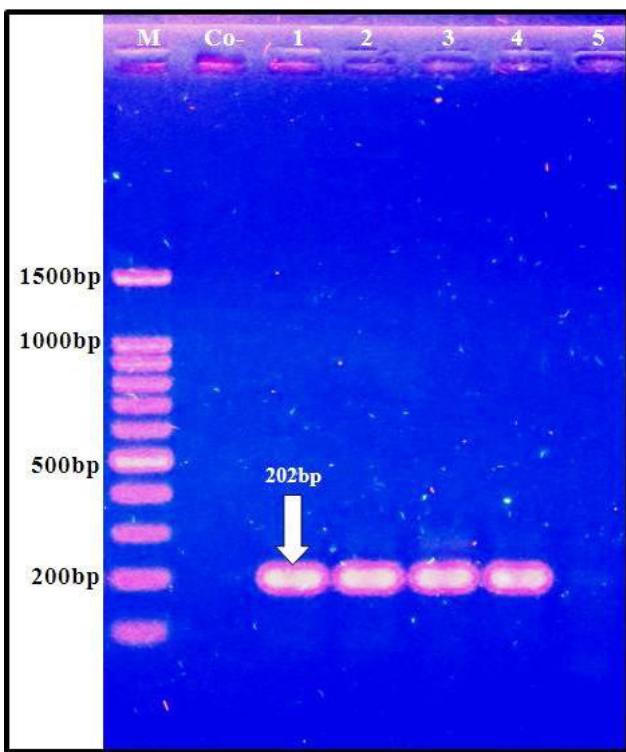


Figure 2. Electrophoresis of PCR products. A serial dilution of DNA (positive control) was amplified with primer F3/B3 resulting in PCR product of 202 bp in length (lanes 1=1:10, 2=1:15, 3=1:20, 4= 1:25 and 4= 1/30). M: 100 bp-plus molecular weight marker, Co-: Negative control (water).

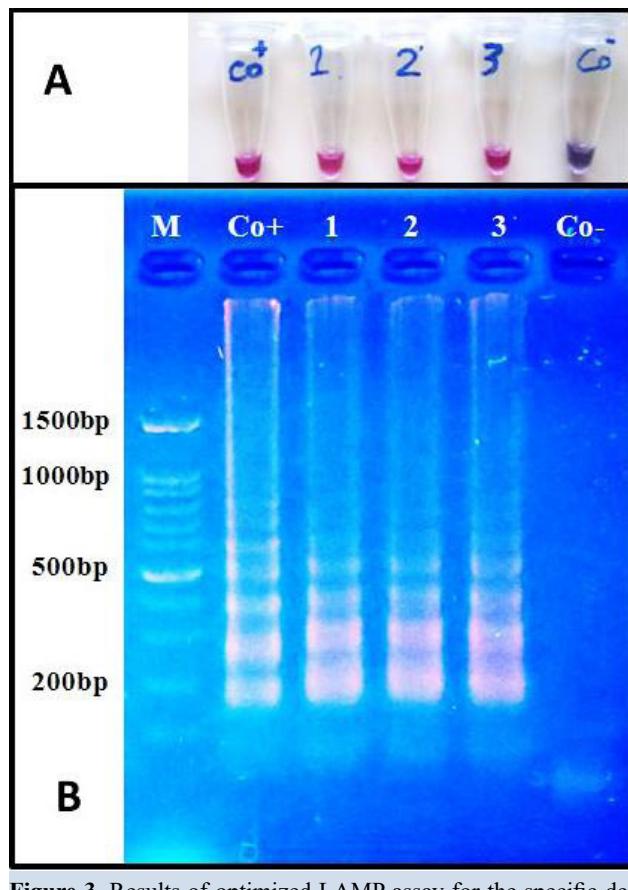


Figure 3. Results of optimized LAMP assay for the specific detection of *T. gondii* DNA targeting the RE gene. (A) Visual detection of the LAMP products based on HNB color change. (B) Agarose gel electrophoresis of LAMP products. M: 100 bp-plus molecular weight marker, Co+: Positive control, Co-: Negative control (water).

Optimization and LAMP assay

Different temperatures (60, 61, 62, 63, 64, 65, 66, 67 and 68 °C), different incubation times (60min, 90 min and 120 min) and different concentrations of MgSo₄ (8mM, 10mM, 12mM and 14mM), Bst DNA polymerase (64, 128, 192, 256, 320 U/ml) and betaine (0.8 mM, 1mM and 1.2 mM) were examined to improve the efficiency of LAMP reactions. The colorimetric assay for LAMP carried out by adding Hydroxy naphthol blue (HNB).

Statistical analysis

Chi-square (χ^2) test was used to compare the variable factors (season, sex, age and type of animal) in animals infected with *T. gondii* by using Statistical Package for Social Services (SPSS Inc, Chicago, USA) version 18.0. A p-value equal to 0.05 or less than 0.05 was considered statistically significant.

RESULTS

Optimization of LAMP reaction

During optimization, the best LAMP assay was performed in a 25 µl reaction volume as follows: 2.5 µl of 10× ThermoPol buffer (New England Biolabs, USA), 4.8 U Bst DNA polymerase (New England Biolabs, USA), 0.8 mM Betaine, 8 mM MgCl₂, 1.4 mM of each dNTP, 1.6 µM of the primers FIP/BIP, 0.2 µM of the primers F3/B3, 0.4 µM of the primers LF/LB and 0.5 µl (1:25 dilution) of DNA as the template. The best results were obtained when the reaction was performed at 66°C and the minimum time for completion of the reaction was 90 min. The reaction was finalised at 80°C for 10 min. HNB was added to the reaction solution at the final concentration of 120 µM. The color changes occurring in the tubes were monitored by naked eye observation. A color change to reddish blue demonstrates a positive LAMP reaction while negative samples stay in violet color (Figure 3-A).

LAMP on the heart samples

The genomic DNA extracted from sheep hearts were tested by LAMP assay as explained in LAMP optimization. LAMP assay revealed that 96.4% (241/250) of samples were positive for *T. gondii* DNA. The prevalence of *T. gondii* was significantly higher in female animals as compared to male animals ($p \leq$

0.05) (Table 2). In spring season a relatively higher prevalence was shown as compared to other seasons, but the difference was not statistically significant ($p = 0.07$). Similarly, no statistically significant difference in *T. gondii* prevalence was noted between the type of animals ($p = 0.16$), and age groups ($p = 0.96$).

Table 2. Prevalence of *Toxoplasma gondii* DNA in sheep of Isfahan province according to the season, sex and age based on the LAMP method.

Category	Level	Number tested	Positive	Prevalence %	95% Confidence Interval		p-value
					Lower	Upper	
All animals	Total	250	241	96.4	94.1	98.7	-
	Winter	133	125	94.0	89.9	98.0	
Season	Fall	50	49	98.0	94.1	101.9	0.07
	Spring	67	67	100.0	100.0	100.0	
Age	<1 Year	54	52	96.3	91.3	101.3	0.96
	1-3 Years	196	189	96.4	93.8	99.0	
Sex	Female	105	104	99.0	97.2	100.9	0.05*
	Male	145	137	94.5	90.8	98.2	

* $P \leq 0.05$

DISCUSSION

Toxoplasmosis caused by *Toxoplasma gondii* is distributed worldwide. The disease is considered an important zoonosis that affects not only humans but also livestock. The prevalence of *T. gondii* is higher in small ruminants as compared to cattle, due to the higher susceptibility of small ruminants to *T. gondii* than cattle (Dubey, 2009; Sharif et al., 2015). Iran is one of the biggest sheep raising and consuming countries (more than 45 million sheep per year) in the world. Economic losses caused by abortions, stillbirths, fetal mummification and births of weak lambs from sheep with toxoplasmosis are predicted to be enormous. Furthermore, these impact on people's lives who are dependent on sheep meat production as a most important source of income (Pan et al., 2017). The prevalence of *T. gondii* in intermediate and final hosts completely differs from one country to another depending on the feeding behavior of animals and humans, environmental factors and socio-economic patterns (Hassanain et al., 2013). Oocysts, bradyzoites, and tachyzoites are the three infectious stages of *T. gondii*. Transmission of tachyzoites can occur via the placenta following primary maternal infection and rarely from ingestion of unpasteurized milk or by direct entry into the bloodstream through a blood transfusion or laboratory accident (Dubey, 2009; Sharif et al., 2015). Approximately 50% of human cases of toxoplasmosis result from the ingestion

of bradyzoites or tissue cysts in meat, meat-derived products or edible offal (such as heart, liver, and kidney) (Hassanain et al., 2013). Lamb meat is one of the major meats used for barbecue (Kebab) in Iran. Because barbecued meat is not cooked properly, there is risk of food-borne toxoplasmosis. For this reason, sheep toxoplasmosis may be the major source of the disease in humans.

In this study, samples were taken from the heart of sheep. This site of sampling was selected because following oral infection of small ruminants, *T. gondii* is mainly localized in the heart and brain tissues (Esteban-Redondo et al., 1999).

A wide range of serological methods including indirect fluorescent antibody tests, enzyme-linked immunosorbent assay, immunosorbent agglutination assay, indirect hemagglutination assay, modified agglutination test, Sabin-Feldman dye test, and Western blot have been applied in extensive epidemiological surveys (Dard et al., 2016). Although serological tests are used extensively in epidemiologic studies, because they are time-consuming, lack in diagnosing primary infections, present antigen cross-reactivity, and antibodies disappear in the long-term, these tests are not reliable when compared to molecular techniques (Sun et al., 2017).

Several diagnostic molecular methods, based on various targets and protocols, have been developed

for direct detection of *T. gondii* DNA in tissues (Khan and Noordin, 2019). The first PCR assay for the detection of *T. gondii* was founded based on the amplification of the 35-repeat B1 gene of the *T. gondii* genome (Burg et al., 1989). Subsequently, various genes including B1-genes, P30, 18S rRNA, the AF146527 element or 529-bp repeat element (RE) have been used for molecular detection of *T. gondii* in different samples (Rostami et al., 2018). Previous studies have shown that the multi-copy genes are more sensitive as compared to single-copy genes for molecular diagnosis of the parasite in samples (Fallahi et al., 2014). The 529 bp-RE genes are repeated 200- to 300-fold in the *T. gondii* genome and exist in all *T. gondii* tested (60 strains) and differentiated the *T. gondii* DNA from the DNA of humans, animals, and the other parasites (Homan et al., 2000). The results of many studies demonstrated that PCR and nested PCR based on the RE gene detected more positive samples as compared with the B1 gene (Fallahi et al., 2014; Homan et al., 2000). Results of PCR with the two multi-copy genes demonstrate that the B1 gene is 10-100 times less sensitive than the 529-bp RE gene in the detection of *T. gondii* (Homan et al., 2000).

The LAMP test is a specific, highly sensitive, and simple method compared to conventional PCR and nested-PCR. This method is not expensive and time-consuming, and it does not require complex equipment. Till now, LAMP has been used for the detection of different parasitic protozoan diseases, including cryptosporidiosis, piroplasmosis, and trypanosomosis (Sun et al., 2017). This method is a favorable molecular technique to be used for the diagnosis of toxoplasmosis, specifically in developing countries with a high prevalence rate (Khan and Noordin, 2019). In a comparative study the sensitivity of the LAMP-based on the 529 bp-RE genes was 1000- and 100-fold higher than that of the RE-nested PCR and B1-LAMP, respectively (Kong et al., 2012).

In the present study, 96.4% of samples were found to be positive for *T. gondii* infection by LAMP. To the best of our knowledge, the current study is the first to use the LAMP assay to determine the prevalence of *T. gondii* in sheep in Iran. Several serological studies have reported the prevalence of *T. gondii* in different regions of Iran, but little research has been performed on molecular detection of *T. gondii* infection in small ruminants. Recent reports in Iran revealed a molecular prevalence of *T. gondii* in sheep and goats in the range of 1.26–66% and 1.07–44.16 %, respectively

(Armand et al., 2016; Asgari et al., 2011; Habibi et al., 2012; Nematollahi et al., 2014; Tavakoli-Kareshk et al., 2017; Tavassoli et al., 2013).

Most of the epidemiological surveys on small ruminants in Iran were established upon detecting *T. gondii* antibodies serum samples. According to those surveys whose methodology allows us to estimate prevalence rates, the overall seroprevalence of toxoplasmosis in sheep and goats of Iran is estimated to be 31% and 27%, respectively (Sharif et al., 2015).

In Iran, the highest seroprevalence rate for toxoplasmosis that was ever reported was 95% in Mazandaran province, northern Iran and the lowest prevalence rate was 5.2% in the northeast of Iran at Khorasan province (Razmi et al., 2010) Iran</style></title><secondary-title>Journal of Parasitology</secondary-title></titles><periodical><full-title>Journal of Parasitology</full-title><abbr-1>J Parasitol</abbr-1></periodical><pages>812-815</pages><volume>96</volume><number>4</number><dates><year>2010</year></dates><isbn>0022-3395</isbn><urls></urls></record></Cite></EndNote>. The prevalence recorded in this study is based on a molecular method and is higher than those previously reported in small ruminants of Iran: 37.5% (sheep) and 22.7% (goat) in southwest Iran (Asgari et al., 2011), 34.32 % (sheep) in south of Iran (Armand et al., 2016), and 56.66% (sheep) and 44.16 (goat) in Eastern Iran (Tavakoli-Kareshk et al., 2017). This prevalence could reflect the LAMP is 10-1000 times more sensitive compared with conventional nested PCR (Khan and Noordin, 2019; Kong et al., 2012).

The high prevalence in sheep (96.4%) reported in the present study could reflect the widespread distribution of the parasite and high level of environmental contamination.

This finding indicates that *T. gondii* oocysts are widely distributed in small ruminants farms in Isfahan. Horizontal transmission of *T. gondii* to small ruminants by the oral uptake of environmentally resistant oocysts through contaminated fodder or water is considered the most important route of transmission (Stelzer et al., 2019). This high prevalence suggesting that *T. gondii* oocysts may be transmitted by water and annual precipitation possibly help the oocyst spread and retains access for potential hosts. Since farm animals represent a direct source of infection for humans, but also a possible reservoir for the parasite, it is important to control *T. gondii* infec-

tions in livestock (Guo et al., 2015).

Measures of hygiene and regimes of cleaning and disinfection applied at the farms may play an important role in the infection of livestock with *T. gondii* because cleaning reduces the probability of contamination of the facilities with oocysts and may also reduce exposure to infected intermediate hosts. The presence of cats often determines the risk of a farm being infected, thus cat control is a key to the control of toxoplasmosis in farms, as well as rodent control (Stelzer et al., 2019). The high prevalence of *T. gondii* in small ruminants is an important source of meat-borne toxoplasmosis in humans and is limited by avoiding the consumption of undercooked meat containing bradyzoites (Dubey et al., 2008).

However, some of the disagreement between the recorded results could be the consequence of (1) the use of inadequately small number of animals, which will lead imprecise estimate and possibly impaired objectivity of data; (2) different geographic areas with various climatic conditions; (3) management practices; (4) different diagnostic methods (Serological or Molecular) with different sensitivity and specificity. Various types of samples and selected cut-offs affect the efficiency of the serological tests; (5) sex and age of animals (Abdallah et al., 2019; Olsen et al., 2019; Sharif et al., 2015); and (6) feeding behavior of animals. In the pasture, goats tend to consume from the top of plants, trees or shrubs to obtain the desired forage and do not like to graze near the soil surface, as sheep do (Guo et al., 2015).

Concerning season, the prevalence in spring was higher than in fall and winter, but statistically, there was no difference significant ($P>0.05$). Our finding is similar to the results of one study in Iran (Armand et al., 2016) and contrary to those who indicated a statistically significant between season and *T. gondii* prevalence (Gebremedhin et al., 2014; Yin et al., 2015). In spring, the climate is temperate and humid which extends the survival time of *T. gondii* oocysts. Also, the increased activity of cats in spring leads to the widespread distribution of oocysts. These may explain the higher seroprevalence in spring.

In several studies, it has been found that the prevalence of the *T. gondii* infection increased with age. This could be due to a shorter exposure of the younger to *T. gondii* infection than the adults (Guo et al., 2015; Sharif et al., 2015; Stelzer et al., 2019). The results of the current investigation suggest that

there was no significant difference in the prevalence between the age groups in Isfahan sheep ($P > 0.05$), which was similar to previous studies (Alvarado-Esquivel et al., 2013; Armand et al., 2016; Yin et al., 2015).

Regarding sex, the prevalence in female animals was higher than in male animals ($p \leq 0.05$). Several studies found that the prevalence in females was higher than males (Abdallah et al., 2019; Asgari et al., 2011; Kamani et al., 2010; Lopes et al., 2010). However, no statistically significant difference between the prevalence in females and males has been reported in some studies (Alvarado-Esquivel et al., 2013; Armand et al., 2016; Khezri et al., 2012; Yin et al., 2015). The higher prevalence in females was probably due to: (1) In Iran, females are reared for breeding purposes and live much longer exposed to *T. gondii* oocysts on the pastures. (2) The immune response is lower in ewes before and after parturition.

CONCLUSIONS

In conclusion, the LAMP assay targeting the RE gene was employed successfully in field samples for the detection of *T. gondii* and is purposed as a suitable method for the routine molecular diagnosis of toxoplasmosis, where providing of modern equipment is not possible. The results of the present study demonstrated a high rate of *T. gondii* DNA in the heart tissue of slaughtered sheep compared with other parts of Iran and the world. In the light of the high rate of *T. gondii* infection in sheep in this region, undercooked eatable tissues of these animals must be considered as sources of *Toxoplasma* infection for humans. Proper cooking of lamb meat and standardization of preparation and handling of meats can be recommended for preventing the transmission of *Toxoplasma* to humans. Moreover, not feeding raw or undercooked meat to cats and keeping cats outside the sheep breeding farms will reduce the risks of infection from cats to sheep.

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CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest.

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