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Original papers

Prevalence of cerebral toxoplasmosis among slaughtered sheep in Semnan, Iran

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ABSTRACT. Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii*. Felids are definitive hosts and all warm-blooded animals can be intermediate hosts. Some animals such as sheep, goats and pigs are sensitive to infection. In sheep production systems, toxoplamosis can cause abortion and economic loss. In public health, this disease can be transmitted to humans by the consumption of undercooked infected meat or other organs. In this study, *T. gondii* DNA was detected by B1 gene amplification in 140 randomly-selected brains of slaughtered sheep in Semnan, Iran. The prevalence of ovine cerebral toxoplasmosis was estimated using 95% confidence interval. The brain was selected as a target organ because it gives the highest detection rates, and the results can be compared with previous data from other countries. Our findings indicate that *T. gondii* is present in ovine tissues and can be passed on to humans by consuming undercooked or raw meat and other organs such as the liver. The infection can be lethal for immunosuppressed individuals and can cause abortion or birth of infected children in pregnant woman.

Key words: *Toxoplasma gondii*, sheep, brain, toxoplasmosis, prevalence, molecular detection

Introduction

Toxoplasma gondii as an obligatory intracellular zoonotic protozoan parasite belonging to the phylum Apicomplexa, and the group of cyst-forming coccidians. There are two phases in its life cycle: a sexual phase which takes place in domestic cats and other felids [1,2] and a non-sexual phase taking place in an intermediate host, this being any warm-blooded animal. The parasite has three infectious forms of the parasite: oocysts containing sporozoites excreted in cat faeces, tachyzoites (the rapidly dividing form) and cysts containing bradyzoites (the slowly dividing form) in intermediate hosts [3].

Sheep are sensitive intermediate hosts for toxoplasmosis. They are infected by consuming water and food contaminated with oocysts. Tissue cysts are then formed in different organs such as the central nervous system (CNS), kidney, heart, muscle tissue, liver and lungs [4]. Ovine toxoplasmosis infection has two key consequences. First, *T. gondii* can cause abortion among ewes and hence economic loss [5,6] and second, meat and meat-derived products and other cyst-containing tissues can be sources of infection for humans if undercooked [4].

Cases of ovine toxoplasmosis have been reported from all around the world [7], but some important studies in Iran have found the disease to be emerging. Ovine abortion caused by *T. gondii* has been estimated to be 13.5% by PCR, and 9.5% by histopathology and immunohistochemistry methods [8,9]. An ovine abortion outbreak was reported in a one flock [10] and other studies have identified the parasite in different sheep tissues [11–14].

The most important sources of human infection is undercooked food contaminated with oocysts, such as meat and other animal products such as liver, vegetables and water; in addition, infection can result from the accidental ingestion of oocysts that have been excreted by infected cats and cross-contamination during food preparation [15–19]. Toxoplasmosis can be a life-threatening condition among immunosuppressed individuals and can also cause abortion in pregnant women if they are

infected for the first time during pregnancy [20]. In the USA, 131 mothers who had given birth to children infected with *T. gondii* were recalled and 50% of them had eaten undercooked meat [21].

The DNA of *T. gondii* as an obligatory intracellular parasite can be detected in brain, skeletal and cardiac muscles, liver, spleen, lung and placentum but brain is the most frequently infected tissue [22–25]. Molecular examination is one of the most sensitive tests for *T. gondii* detection. Among different molecular techniques for *T. gondii* DNA detection, the nested PCR assay on B1 gene (35 copies per parasite) which is one of the most sensitive and specific tests [26–28]. Unfortunately, the Veterinary Faculty of Semnan, Iran has not been equipped by real time PCR instruments but there are some new quantitative tests for the parasite DNA detection [29–31].

The aim of this study is to determine the prevalence of cerebral toxoplasmosis among slaughtered sheep in Semnan, Iran using molecular methods.

Materials and Methods

A total of 140 sheep brains were randomly collected in sterile tubes from slaughtered sheep in Semnan industrial abattoir, Iran during three months in 2016 (October – 46 samples, November – 46 and December – 48). In the Parasitology Laboratory, each sample was homogenized. Tris-Cl (pH 8.0) was then added to 100 mg of brain tissue, and the mixture was treated with 200 mg/ml proteinase K (Fermentas®, Lithuania) for three hours.

DNA was extracted by the phenol-chloroform method and the purified DNA was resuspended in TE (10mM Tri sans 1mM EDTA, pH 8.0) and stored at -20°C. An RH strain of *T. gondii* provided by Zajan University of Medical Sciences, Zanjan, Iran, was used as a positive control, while distilled water was used as a negative control.

DNA samples were examined by nested PCR on B1 gene amplification, using two sets of oligonucleotide primers as described by Burg et al. [26]. The DNA and primers were added into 20 µl reaction volumes the Accupower PCR premix kit (Bioneer®, South Korea) with a final concentration of each dNTP of 250 µM in 10 mM Tris-HCl pH 9.0, 30 mM KCl and 1.5mM MgCl2, 1 U Taq DNA polymerase. 10pmol of each PCR primer (Takapouzist Co. Iran) and 1µl of DNA template (250–500 ng) were added to each reaction and the remaining 20 µl reaction volume was filled with sterile distilled water. The reaction cycle was as follows: 94°C for 3 min, 40 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 7 min. The first PCR product was diluted 1:10 and the nested PCR reaction condition was then performed under the same conditions as the first round, except for the annealing temperature was performed at 52°C, and the number of cycles was 30 [8]. A 193 bp PCR product should be obtained from the first amplification; however in the present study, all 140 samples were then subjected to the second round of nested PCR, regardless of the presence or absence of a visible band; the second PCR product was electrophoresed through 1.5% agarose gel and the resulting 96 bp band was visualized under UV. A 100 bp molecular marker was used for size evaluation (Fermentas®, Lithuania), Fig.1.

The infection rate of the parasite in different months was analyzed by Chi-Square Test, SPSS software.

Results

T. gondii DNA was detected in 26 out of 140 (18.57%) sheep brain samples. Thus, the prevalence

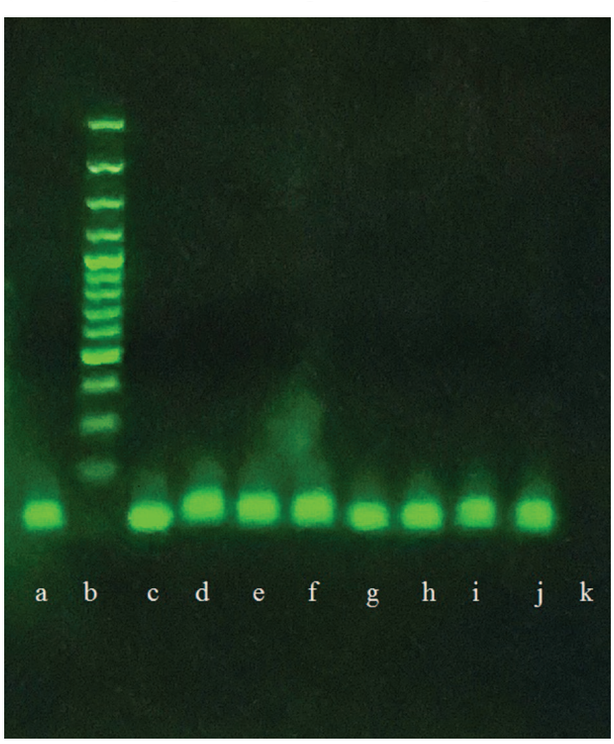

Fig.1. *Toxoplasma gondii* DNA amplification on B1 gene
a: positive control, b: 100bp plus molecular marker, c-j: positive samples, k: negative control

of cerebral toxoplasmosis was estimated as 12.1–25% with a 95% confidence interval (CI), Fig. 1. Nine samples (6.43%) was infected on October, six samples (4.28%) on November and eleven samples on December (7.85%). There was no significant difference among the infection rate of *T. gondii* in these three months of the year 2016 (P>0.05).

Discussion

About 50% of toxoplasmosis cases in the USA have been attributed to food transmission of the parasite [32]. Some studies have examined the presence of *T. gondii* in various tissues of sheep, these being a sensitive to the disease and a protein source for human. In Europe viable *T. gondii* was detected in two healthy lambs in England [33]. In an experimental study, *T. gondii* DNA was detected in eight out of 12 infected sheep, with the parasite being observed at higher levels in the brain and heart tissues than other muscle samples [22]. *T. gondii* was isolated from five slaughtered sheep in Denmark [34]. Elsewhere, infected tissues were identified in of 6% out of 150 studied sheep [35]. In one study in the USA, 77.9% of 68 lamb hearts were found to be positive for *T. gondii* infection [36]. In Brazil, *T. gondii* DNA was detected in 21.43% of CNS samples and hearts from sheep [37], and in 31 and 32% of muscle samples taken from slaughtered ewes in two abattoirs [39]. In Moracco, microscope examination revealed the presence of tissue cysts in 30% of 50 examined brain samples [25].

One study in Fars, Iran, found *T. gondii* DNA in 37.5% of 56 examined sheep. DNA was detected in tongue and brain more frequently than femoral and intercostal muscles [12]. Elsewhere in Iran, *T. gondii* DNA was also detected in 14% of muscle tissues of 50 slaughtered sheep in Ahvaz [13], 38% of 50 examined sheep were infected by *T. gondii* n Chaharmahal va Bakhtiari province: 32% were detected in brain, 30% in the liver, 28% in the femur muscle and 16% in tongue samples [40]. In addition, *T. gondii* DNA was detected in 34.32% of 370 ovine muscle samples in Jahrom [14].

In the present study, *T. gondii* DNA was amplified in 18.57% of 140 brain samples (12.1–25%; 95% CI) in slaughtered sheep in Semnan, Iran. There was no data about *T. gondii* prevalence among sheep in Semnan, Iran and the results because of the first information of the infection rate in the area are valuable. In other mentioned studies in Iran the sample size of the studies were less than what was needed for prevalence research (most of them had 50 samples) [12,13,40]. Therefore, they were not comparable with the current study, except in Jahrom [14] study which 34.32% of 370 muscular samples were positive which was about twice higher than our result (18.75%). It can be interpreted that Jahrom in Iran has more humidity than Semnan and the disease may be more prevalent there because of its weather. Another finding of the research is *T. gondii* infection rate among slaughtered sheep in Semnan didn't have significant difference among various sampling months (p>0.05).

Two rounds of PCR assay in nested PCR give sharp and intense bands which increase its sensitivity in comparison with simple PCR but there are some limitations in nested PCR assay that one of the most important one is cross-contamination. In this research, it has been minimized by using prepared reagent, Accupower PCR premix kit (Bioneer®, South Korea) and negative controls in each PCR experiment.

Free-range approaches to raising small ruminants increases the risk of infection with *T. gondii* [19], but food-borne transmission of infection can be easily prevented by adequate cooking and freezing. Utensils and hands should be rinsed with hot water after contact with raw meat and vegetables [19]. Unfortunately, in some parts of Iran, raw and undercooked liver is consumed according to local customs.

When cooking meat, the internal temperature should be increased to 67°C [41] and 71.1°C for ground meat [43] but microwave cooking may not kill *T. gondii* [43].

Different optimal times and freezing temperatures have been reported for killing *T. gondii.* Studies have reported the parasite being killed by being kept at at –12°C internal temperature for more than two days [44] and by –7 to –12°C for four days [45]. According to Djurkovic-Djakovic and Milenkovic [46], at least three days at –20°C is necessary to kill *T. gondii* bradyzoites [46]. Gamma irradiation and high pressure can also be used to kill *T. gondii* but these methods change the color and texture of the meat [45,47,48]. Increased survival of *T. gondii*, for six weeks at 4°C, was observed in vacuum-packed meat containing *T. gondii* tissue cysts [49].

Some studies have examined the effects of salt concentration and acidity (pH) on *T. gondii*

infectivity. *T. gondii* cysts in mouse brains exposed to different NaCl salt concentrations survived more [50]. Muscle cysts of *T. gondii* were found to survive for eight days at more than 2% NaCl concentration, but for 4 days in 2% nitrite. *T. gondii* cysts have high pH tolerance and can live for up to 26 days at pH 5.0 [51].

As mentioned above, toxoplasmosis is one of the most important parasitic infections among sheep and human. Free ranging production systems make disease control difficult in small ruminants but knowledge and training can easily prevent infection occurring among the human population, which is especially important among pregnant women and immunosuppressed individuals. According to the transmission routes of *T. gondii* it is also recommended that meat and meat fed to carnivorouse pets are also well cooked.

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