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Short Communication

Molecular Survey of *Toxoplasma gondii* in Sheep, Cattle and Meat Products in Chaharmahal va Bakhtiari Province, Southwest of Iran

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Abstract

Background: Toxoplasmosis is a worldwide spread disease. The present study examined the prevalence of *Toxoplasma gondii* infection among animals of edible meat (cattle and sheep) in Chaharmahal va Bakhtiari Province (Southwest of Iran) in 2012. Furthermore, we attempted for the first time to identify this parasite from the meat products in the province.

Methods: The tongue, brain, femur muscle and liver of 50 sheep and 70 cattle as well as 50 samples of meat products were selected and collected to perform molecular survey using Nested-PCR method.

Results: Of the studied sheep, 38% were infected. The infection rate in the age groups under 1 year, 1-2 years, and more than 2 years was 25%, 35.29% and 52.94%, respectively. The infection rate in femur muscle, brain, liver and tongue was 28%, 32%, 30% and 16%, respectively. Of the studied cattle, 8.57% were infected. The infection rate in the age groups 1-2 years, 2-4 years, and more than 4 years was 3.7%, 9.09% and 14.28%, respectively. Sheep was infected 6 times more than cattle (OR = 6.53 CI = 2.374-18.005). The infection rate among samples of meat products was 12% (6 samples out of 50 samples).

Conclusion: Due to the high rate of this parasitic infection among the slaughtered animals as well as meat products in this region, the use of infected material can be one of the main risk factors of transmission of the parasite to humans.

Introduction

Diseases transmissible between humans and animals are one of the major public health problems in many countries. One of these diseases is toxoplasmosis. The cause of disease is an obligatory intracellular parasite with a worldwide spread called *T. gondii* (1). It is one of the most prevalent apicomplexan parasites found in various animal species and humans. Members of Felidae, especially cats, serve as the final hosts of the parasite, while humans and warm-blooded animals, including cats, play a role as intermediate hosts (2).

In regions where cats are more accessible or people eat more raw and undercooked meats, the infection rate is high (3). The use of undercooked meat in pregnant women is one of the most important factors of *Toxoplasma* infection during pregnancy. The high infection rate with this protozoan during pregnancy can lead to miscarriage of livestock and humans (4-5). Sheep and cattle in most countries are of great economic importance, because they are a source of human nutrition. *T. gondii* in sheep and cattle is considered as a source of infection for humans and other carnivores (6).

One of the most important potential sources of human toxoplasmosis is chronically infected cattle. Therefore, it is necessary to investigate the prevalence of *T. gondii* in the animals whose meat is used by humans (7). *T. gondii* infections are diagnosed by isolating the organism, serologic tests, polymerase chain reactions and demonstration of tachyzoites in tissues and body fluids by histological methods (8). The seroprevalence of toxoplasmosis in the northern, southern and all parts of Iran have been 55%, 29% and 51.8%, respectively (9).

Molecular method is one of the most important and the best methods to detect protozoan in tissue of infected animals. The advantages of molecular method include the

need for low genetic material, the lack of confounding effects of environmental conditions and host, the examination of many samples in a short time and the high sensitivity of the test.

In this study, due to high sensitivity of molecular method in *T. gondii* detection, Nested-PCR method was used for detection of this protozoan in sheep and cattle tissues. Furthermore, the identification of *T. gondii* in meat products was attempted for the first time by molecular method. Chaharmahal va Bakhtiari Province and Shahrekord with large numbers of sheep and cattle is considered as one of the important areas in Iran in the field of meat production and tissue cysts of *T. gondii* in meat and viscera of sheep must be considered as important sources of infection in human.

Materials and Methods

Study area

Shahrekord is the capital of Chaharmahal va Bakhtiari Province and is situated in the north of the Zagros Mountains. Shahrekord has a cold semi-arid climate with hot summer days, mild summer nights, cool winter days and cold winter nights. The province is mountainous with an average elevation of 6790 feet above sea level.

Sampling

To perform molecular survey, the samples of tongue, brain, femur muscle and liver of 50 sheep and 70 cattle were collected and transferred to the laboratory referring to the Jonghan industrial slaughter house. Moreover, 50 samples of fermented sausages were collected from local grocery stores in shahrekord. The tissue samples were stored at -80 °C until work up.

DNA extraction

Tissue samples (TSs) of approximately 1 g were thawed, minced with a sterile blade and transferred to sterile bags containing 6 ml of

Tris-EDTA (TE). Samples were homogenized in a stomacher for 3 min and then stored at -80 °C in 400 µl aliquots that were thawed prior to DNA extraction. Proteinase K (final concentration of 200 µg/ml) and sodium dodecyl sulfate (SDS) (final concentration of 1%) were added to each tube containing TSs and incubated at 56 °C, for 1 h, shaking every 10 min. The digestion procedure was completed by incubating the samples at 96 °C, 10 min. A phenol-chloroform-isoamyl alcohol solution (25:24:1, v: v: v) was added to each sample (1:1, v: v) and mixed. After centrifugation at 12000g at 4 °C for 10 min, the aqueous phase was transferred to another tube and an equal volume of chloroform-isoamyl alcohol (24:1) was added to the sample. Following the second run centrifugation (the same conditions as above), the aqueous phase was recovered again and the DNA was precipitated by at least 1h of incubation at -20 °C with 3M sodium acetate and 100% ethanol (10:1:20, v: v: v). The DNA was then pelleted by centrifugation at 13000g for 20 min, washed twice with 75% ethanol, dried for at least 30 min at room temperature and resolved in 100 µl of pre-heated ultrapure sterile water (10).

Assessments of DNA concentration and purity

DNA concentration was determined by measuring the absorbance at 260 nm. Its purity was defined as the ratio of absorbance at 260 to 280 nm wavelengths by using a spectrophotometer BioPhotometer (Eppendorf, Milan, Italy).

PCR amplification

The technique employs two pairs of primers, the external ones (with a higher melting temperature, T_m) in a lower concentration and the internal primers (with a lower T_m) in a higher concentration to prevent the interference between the first and second amplification rounds. Therefore, two oligonucleotide primer pairs were used. The external primers were GRA6-R1 (5'-GCACCTTCGCTTGTGGTT -

3') and GRA6-F1x (5'-ATTTGTGTTTCCGAGCAGGT-3') producing an amplified product of 546 bp. The internal primers were GRA6-R1x (5'-TCGCCGAAGAGTTGACATAG -3') and GRA6-F1 (5'- TTTCCGAGCAGGTGACCT-3') that amplified a region of 351 bp of the *T. gondii* (15). The first 50 µl PCR reaction mixture contained outer primers at a final concentration of 50 pmol for each primer pair, 20 mmol/L dNTPs and 1U recombinant taq DNA polymerase in 1×PCR reaction buffer (50 mmol/L KCl and 10 mmol/L Tris-HCl, 1.5 mmol/L Mg₂Cl₂, 0.1% triton×100; Sinagen Co., Iran). The PCR amplification was performed for 4 min at 94 °C for one cycle, followed by 35 cycles using denaturation at 94°C for 45 sec, annealing for 45 sec at 55°C, extension for 1 min at 72 °C and the final extension for 10 min at 72 °C. The nested PCR reaction was performed using 5 µl of the first PCR reaction in a mixture containing the inner primers at the final concentration of 50 pmol for each, 20 mmol/L dNTPs, 1.25 U recombinant taq DNA polymerase in 1×PCR reaction buffer. Amplification was carried out at 94 °C for 4 min (one cycle), then followed by 35 cycles using denaturation at 94 °C for 45 sec, annealing for 45 sec at 55 °C and extension for 2 min at 72 °C. The run was terminated with a final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis through 1.5% (w/v) agarose gels in TBE buffer containing 0.05% ethidium bromide, and the bands were visualized under UV light on a transilluminator (11).

Data Analysis

The results were analyzed with statistical package of SPSS (version 18), using Chi-square test. A *P*-value of <0.05 was set as statistically significant.

Results

The A₂₆₀/A₂₈₀ absorbance ratio ranged 1.7 to 1.97. Of the studied sheep, 38% were infected. The infection rate in the age groups

under 1 year, 1-2 years, and more than 2 years was 25% (4 of 16 samples), 35.29% (6 of 17 samples) and 52.94% (9 of 17 samples), respectively. A direct significant correlation was observed between age increase and infection rates ($P = 0.036$, $R = 0.298$). The infection rate in femur muscle, brain, liver and tongue was 28%, 32%, 30% and 16%, respectively. No significant direct correlation was observed between the liver and femur muscle infection ($P = 0.542$, $R = 0.092$). The highest infection rate was found in tongue and brain ($P = 0.001$, $R = 0.674$), and the tongue and femur muscle ($P = 0.001$, $R = 0.661$), respectively. Of the studied cattle, 8.57% were infected. The infection rate in the age groups 1-2 years, 2-4 years, and more than 4 years was 3.7%, 9.09% and 14.28%, respectively. There was no significant correlation between age and infection rate in cattle. The infection rates in femur muscle, brain, tongue and liver was 5.71%, 5.71%, 4.28% and 2.85%, respectively. There was a significant direct correlation between the infection rates in all above tissues.

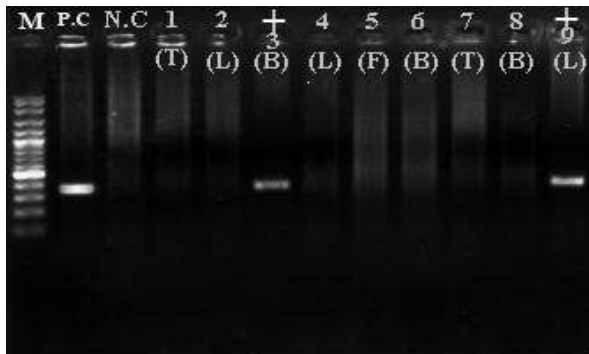


Fig. 1: Agarose gel electrophoresis of Nested-PCR products of tissue samples of sheep, Lane M represents 100-bp DNA ladder. Lane P.C is for positive control. Lane N.C for negative control. Lane 3 and 9 are positive samples. (F: femur muscle, B: brain, T: tongue, L: liver)

The most significant correlation was observed between tongue and femur muscle infection ($P = 0.001$, $R = 0.556$), and the brain and tongue infection ($P = 0.001$, $R = 0.47$),

respectively. Sheep was infected 6 times more than cattle ($OR = 6.53$ $CI = 2.374-18.005$). The infection rate among samples of meat products was 12%. The results of PCR amplification and Nested-PCR products of tissue samples are shown in Fig. 1-3.

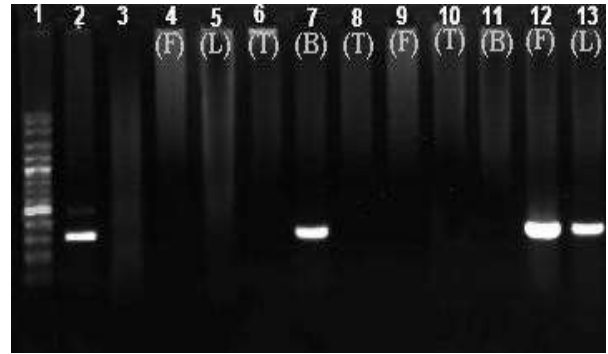


Fig. 2: Agarose gel electrophoresis of Nested-PCR products of Tissue samples of cattle, Lane 1 represents 100-bp DNA ladder. Lane 2 positive control, Lane 3 negative control, Lane 7, 12, 13 positive samples. (F: femur muscle, B: brain, T: tongue, L: liver)

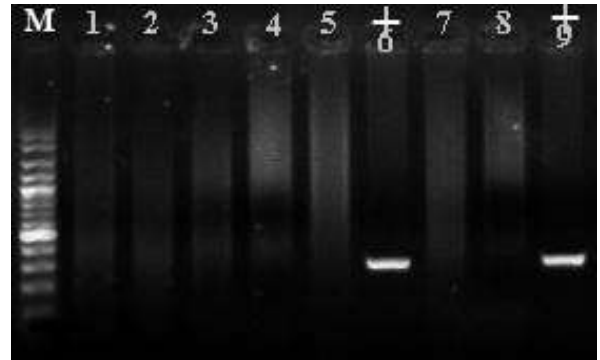


Fig. 3: Agarose gel electrophoresis of Nested-PCR products of meat products, Lane M represents 100bp DNA ladder. Lane 6 and 9 show positive samples

Discussion

Nowadays, toxoplasmosis is considered as one of the most important food-borne diseases (12). The use of infected tissues of cattle and sheep can transmit the disease to humans (13). Our results showed that 38% of sheep

were infected. The maximum and minimum rate of infection was observed in the brain (32%) and the liver (16%), respectively. This finding was consistent with the results of Asgari et al. who found that the rate of *T. gondii* infection in sheep and goats was 37.5% and 22.7%, respectively. Moreover, the infection rate in tongue tissue was higher than other tissues (14). Bonyadian et al. conducted a study on sheep in Chaharmahal va Bakhtiari Province. The seroprevalence infection rate of sheep was 29.1% as obtained by IFT test (15). The results of the present study showed that 8.57% of cattle in the region were infected by *T. gondii*. The highest and lowest infection rate was observed in brain (5.71%) and femur muscle (5.71%) and the liver (2.85%), respectively. The cattle had far less infection than sheep. The lower rate of infection could be attributed to relative resistance of cattle against *T. gondii*. Moreover, a cattle grazing is not free as sheep (16). According our findings, the sheep is likely infected by *T. gondii* 6 times more than cattle. This result was consistent with the results of another study (17). The seroprevalence of toxoplasmosis in cattle in 5 regions of the Fars province was obtained at 20.24% using IFT test (18).

Berger et al. examined the infection rate of meat of animals used as a food source in Switzerland using PCR-RFLP method. The infection rate in cattle, sheep and pigs was 2.7%, 4% and 2.2%, respectively (19). Ergin et al. in Turkey found that the infection rate of cattle brain and muscle was 2 and 6 percent, respectively. The infection rate of sheep brain and muscle was 15 and 20 percent, respectively. However, the infection rate of cattle and sheep was obtained at 24 and 25 percent, respectively using serological methods (20).

The prevalence of *T. gondii* among sheep and cattle shows high discrepancy in various studies. It seems that this variation is due to geographical location, type of food, type of test, measurement methods and the sample size. *T. gondii* DNA was detected in 12% of

these products. This demonstrates the use of infected meat in this product.

The present study is the first attempt to identify *T. gondii* from meat products by molecular method in Chaharmahal va Bakhtiari Province. Mendonca et al. examined 70 samples of meat products in Brazil using PCR and Bioassay methods. No parasite was isolated, while there were 33 positive samples in PCR method (21). In a study conducted in Turkey, 100 meat products prepared by fermentation were examined using molecular method. The infection rate of meat products have been reported at 19% (20).

Conclusion

Given the high rate of *T. gondii* infection in tissues of sheep and cattle, appropriate and effective preventive measures should be done to prevent the transmission of this parasite to humans. It is necessary to avoid eating the raw and undercooked tissues of infected animals. Furthermore, the observation of animal health standards and standardization of methods for the preparation of meat and meat products is of great importance.

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