Original Article

Molecular Identification and Frequency of Cyst-Forming Coccidia (*Sarcocystis, Toxoplasma gondii*, and *Neospora caninum*) in native slaughtered cattle in Kashan, Central Iran

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Abstract

Aim: Cattle is one of the main sources of food supply chain for humans in most countries. The present study aimed to identify the infection rates for *Toxoplasma gondii*, *Neospora caninum*, and *Sarcocystis* spp. by polymerase chain reaction (PCR) method in native slaughtered cattle in Kashan, central Iran**. Materials and Methods:** Totally, 159 diaphragm, esophagus, and muscle samples (53 samples of each) were collected from native beef cattle from Kashan slaughterhouse, central Iran. The genomic DNA was extracted, and PCR method was used separately for detection of *N. caninum*, *Sarcocystis*, and *T. gondii* species using specific primers. **Finding:** *Sarcocystis* was found in 84.9% of muscles, 83% of esophagus, and 84.9% of diaphragm samples. Mixed infection (*Sarcocystis cruzi–Sarcocystis hominis*) was the most common infection, followed by *S. cruzi* and *S. hominis*. *Sarcocystis hirsuta* was not detected in any samples. *T. gondii* was detected only in three (5.7%) out of the 53 muscle tissues samples of cattle. *N. caninum* was found in 18.9% of muscles, 24.5% of esophagus, and 28.3% of diaphragm samples. One of the cattle had coinfection to *Neospora*, *Toxoplasma*, and *Sarcocystis* in muscles simultaneously. There was no statistically significant difference between infection rates and age as well as sex in each organ. **Conclusion:** This study revealed a low prevalence rate of *T. gondii*, but a high prevalence of infection to *N. caninum* and *S. cruzi* or mixed infection of *S. cruzi* with *S. hominis* among slaughtered cattle. Prevention measures such as keeping away dogs from cattle grazing are recommended.

Keywords: Cattle, Iran, Kashan, *Neospora caninum, Sarcocystis, Toxoplasma gondii*

Introduction

Toxoplasma gondii, *Sarcocystis* spp., and *Neospora caninum* are obligate intracellular and cyst-forming apicomplexan parasites that belong to the family Sarcocystidae and subfamily Toxoplasmatinae.[1] These are related coccidian organisms with complex heteroxenous life cycles and different degrees of clinical and economic importance in animals and humans.

Cattle as the meat-producing animal is one of the main sources of food supply chain for humans in most countries, and can be infected with various significant parasitic diseases, of which some are zoonosis. Infections by *N. caninum*, *T. gondii*, and at least three species of *Sarcocystis* (*Sarcocystis cruzi*, *Sarcocystis hirsuta*, and *Sarcocystis hominis*) have a worldwide distribution in cattle.[2,3]

N. caninum and *T. gondii* are two major causative agents of reproductive disorders in ruminants especially cattle.[4] Cattle may get infected by these organisms through ingestion of contaminated water or food to the sporulated oocysts or sporocysts excreted in the feces of definitive hosts.[5]

T. gondii and certain species of *Sarcocystis (S. hominis and Sarcocystis heydorni)* are important parasitic zoonoses

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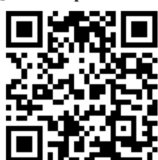

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transmitted to humans beings by ingestion of raw meat from intermediate hosts, whereas the disease caused by *N. caninum* is not considered zoonotic despite serological evidence in humans.[6]

It was established that over 90% of adult cattle have been infected with *Sarcocystis* spp. in many countries.[3] The most prevalent *Sarcocystis* species infecting cattle is *S. cruzi* that causes acute systemic illness, poor growth, and, occasionally, abortion.[3,7]

Results of many available studies conducted worldwide have revealed that coinfection with more than one *Sarcocystis* spp. in muscle of cattle is frequent. Mixed infection of *S. cruzi* with the well-known human pathogen *S. hominis* in cattle is a common occurrence globally. Humans can get infected as final hosts after consumption of undercooked beef harboring mature *sarcocysts* of *S. hominis*. Human infection appears to be mild or asymptomatic, but many infections are mainly characterized by gastrointestinal discomforts such as diarrhea, vomiting, stomachache, and nausea.[8,9]

The prevalence of *S. hominis* in cattle was reported between 6.2% and 97.4% in Europe, Asia, and South America.[10-12] A high prevalence of *S. hominis* (57.5%) had been reported in slaughtered cattle at Yazd, central Iran, using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method.[9]

T. gondii may infect humans and a variety of domestic and wild animals including cattle. Cattle and other farm animals could serve as intermediate hosts for *T. gondii* and could be the sources of human infection.[13] A survey on food and environmental risk factors for acute toxoplasmosis in pregnancy showed that between 30% and 63% of human infections can be attributed to the consumption of undercooked or raw meat products.[14]

A study on the seroprevalence of *T. gondii* in cattle in the state of Pará, Brazil, demonstrates high levels of anti-*T. gondii* antibodies (34.48% and 44.14%) in cattle using Elisa and IFA methods, respectively.[15] The crude seroprevalence rate of *Toxoplasma* infection in cattle in Iran was estimated at 18.1%.[16]

T. gondii infection in cattle usually appears to be asymptomatic and does not seem to be an important cause of abortion among this animal. Although the role of beef consumption is unclear in the transmission of infection to humans, it may be one of the sources of infection among humans due to the large amount of beef consumed.[17]

N. caninum infects a wide range of wild and domestic animals and primarily cattle. It is of significant importance in veterinary medicine. Neosporosis in bovine is now recognized as one of the leading causes of abortions, neonatal mortality, stillbirth, and decreasing milk production.[18]

Majority of data on the prevalence of *N. caninum* infection in cattle in many parts of the world including Iran are based on seroprevalence. The seroprevalence of *N. caninum* in cattle in Iran has been estimated to be around 3.8%–76.2% across the country.[19]

Due to lack of molecular information, the objective of the present study was to determine the prevalence of *T. gondii*, *Sarcocystis* spp., and *N. caninum* and identification of species of *Sarcocystis* by molecular methods, in slaughtered cattle in Kashan, central Iran.

Materials and Methods

Kashan is a city located at the center of Iran, north of Isfahan province, between 50° 55' and 52° 29' east longitude and 33° 30'-34° 27' north latitude. The total area is 4415.07 km^2, with a population of nearly 400,000 people, and has two relatively different climates: moderate climate in mountainous part and hot and arid in deserts. The average rainfall is around 45.61 mm; the mean temperature is 19°C, which can reach as high as 50°C.

This cross-sectional study was carried out from April to September 2020 in Kashan, central Iran. Totally, 159 samples were randomly collected from the muscles, diaphragm, and esophagus (approximately 50 g in weight, free of fat and connective tissue) from native cattle (53 samples from muscles, diaphragm, and esophagus) from the slaughterhouse of Kashan. Twenty grams of each sample was minced by an electric meat grinder and stored at −20°C until DNA extraction.

The genomic DNA extraction was performed from 20 mg of each sample using a DNP™ kit (Cinnagen, Iran), according to the manufacturer's instruction protocol. The extracted DNA was stored at −20°C for the next PCR amplification.

For PCR-based diagnosis of *Sarcocystis* spp., a multiplex PCR protocol was performed as previously described by Chiesa *et al*. (2013) with minor modifications.[20] Multiple sequence alignment was conducted based on 18S rRNA gene sequences in the cattle *Sarcocystis* species to identify regions for designing primers (CLC main workbench was used to survey the sequences, and Primer-BLAST software was used to check the specificity of primers). Three forward primers specific for *S. cruzi* (5'-ATCAGATGAAAATCTACTACATGG-3'), *S. hominis* (5'-ACAGAACCAACACGCTC-3'), and *S. hirsuta* (5'-CATTTCGRTGATTATTGG-3') and a reverse primer (5'-AACCCTAATTCCCCGTTA-3') common to all the three species were used. These primers amplify a 284, 182, and 108 bp fragment for *S. cruzi*, *S. hominis*, and *S. hirsuta*, respectively.

PCR was achieved at a total volume of 20 µL, using commercial ×2 PCR reaction mixture (Pishgam, Iran). Depending on DNA concentration, 1–3 µL of DNA was added to the PCR reaction mixture and amplified in an automated PCR machine (Flexcycler 2, Germany).

The PCR conditions were as follows: an initial denaturation step at 94°C for 5 min and 35 cycles at 94°C for 45 s

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(denaturation), 59°C for 45 s (annealing), and 72°C for 60 s (extension) with a final extension step for 10 min at 72°C.

For *N. caninum* DNA detection, separate PCR was performed with the specific primer pair Np6plus (5'-CTC GCC AGT CAA CCT ACG 93 TCT TCT-3') and Np21plus (5'-CCC AGT GCG TCC AAT CCT GTA AC-3') that amplifies a 328–340 pb amplicon of Nc-5 gene.[21-23]

The 35-fold repetitive and highly conserved B1 gene was selected for PCR of *T. gondii*. Primers Tg1 (5'-AAA AAT GTG GGA ATG AAA GAG-3') and Tg2 (5'-ACG AAT CAA CGG AAC TGT AAT-3') amplifying a 469-bp DNA fragment were used in the present study.[24,25]

The PCR reaction and condition were similar to that of *Sarcocystis* with the exception that the annealing step was performed at 60°C and 57°C for 40 s for *N. caninum* and *T. gondii*, respectively. Five µL of each PCR product was separated by electrophoresis on 1.5% agarose gel, stained by ethidium bromide, and then visualized under ultraviolet light to evaluate the success of the reaction.

For analyzing the data, SPSS v. 17 (SPSS Inc., Chicago, IL, USA) was used and analyzed by Chi-square test and Fisher's exact test. $P < 0.05$ was considered significant.

Ethics statement

This study was approved by the Research Ethics Committee, Kashan University of Medical Sciences, Iran (Ethics code: IR.KAUMS.MEDNT.REC.1398.070).

Results

Overall, *Sarcocystis* was found in 84.9% (confidence interval [CI]: 75.4–94.6%) of the muscles, 83% (CI: 72.9–93.1%) of the esophagus, and 84.9% (CI: 75.4–94.6%) of the diaphragm samples.

The results related to the prevalence of *Sarcocystis* species according to organ samples are reported in Table 1. Mixed infection (*S. cruzi-S. hominis*) was the most present infection, followed by *S. cruzi*, with 34%, 32.1%, and 13.2% of esophagus, diaphragm, and muscle samples, respectively. *S. hirsuta* was not detected in any samples [Figure 1].

A Chi-square test was performed to examine the relationship between gender and infection to *Sarcocystis* sp. The relation between these variables was not significant. The average age of cattle was 2.49 ± 0.04 years (age range: 1–5.5 years). Overall infection was higher in cattle under 3 years old compared to that of over 3 years, but there was no statistically significant difference between infection rates and age ($P > 0.05$).

Infection to *T. gondii* was observed neither in esophagus-, nor in diaphragm-amplified DNA samples. A 469-bp amplicon corresponding to *T. gondii* was detected only in DNA samples in three (5.7%) out of the 53 muscle tissues of cattle [Figure 2]. In the present study, Two 3- and 2-year-old female and one 1-year-old male cattle were positive for *T. gondii*. All of the 3 cattle positive to *T. gondii* were also positive for *Sarcocystis* sp.

Out of 159 slaughtered cattle samples examined for *N. caninum* DNA, 38 (23.9%) (CI: 17.5%–30.3%) were positive for 328–340 pb amplicon of Nc-5 gene. *N. caninum* was found in 18.9% (CI: 8.4–29.4%) of the muscle, 24.5% (CI: 12.9–36.1%) of the esophagus, and 28.3% (CI: 16.2–40.4%) of the diaphragm samples [Figure 3]. One of the cattle had coinfection to *Neospora*, *Toxoplasma*, and *Sarcocystis* in muscle simultaneously. There was no statistically significant difference between infection rates and age as well as infection and sex in each organ ($P > 0.05$).

Discussion

In the present study, 84.9% of the muscles and diaphragm and 83% of the esophagus samples of cattle slaughtered were found positive for *Sarcocystis*. The prevalence of *Sarcocystis* infection in cattle is high in many countries around the world.[26] Published studies using microscopy or molecular techniques

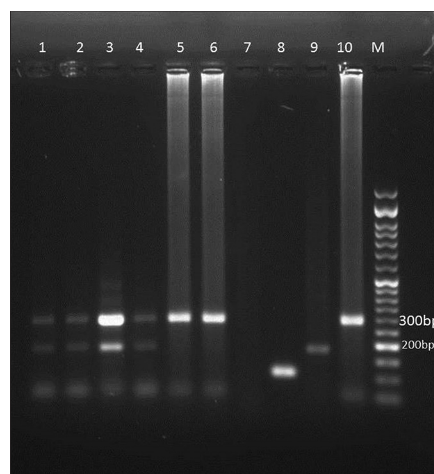

Figure 1: 1.5% Agarose gel electrophoresis of the polymerase chain reaction product of Sarcocystis sp. M: 50-bp DNA ladder. Lane 1–4 *Sarcocystis cruzi* mixed with *Sarcocystis hominis*, lane 5–6: *Sarcocystis cruzi*, lane 7 negative control, lane 8–10 *Sarcocystis hirsuta*, *Sarcocystis hominis* and *Sarcocystis cruzi* positive control, respectively

Table 1: Frequency of sarcocystis species in slaughtered native cattle according to organ samples

Infection samples	*S. cruzi*, *n* (%)	*S. hominis*, *n* (%)	*S. hirsuta*, *n* (%)	*S. cruzi-S. hominis*, *n* (%)	Total, *n* (%)
Muscles	7 (13.2)	0	0	38 (71.7)	45 (84.9)
Esophagus	18 (34)	6 (11.3)	0	20 (37.7)	44 (83)
Diaphragm	17 (32.1)	4 (7.5)	0	24 (45.3)	45 (84.9)

S. cruzi: Sarcocystis cruzi, *S. hominis: Sarcocystis hominis*, *S. hirsuta: Sarcocystis hirsuta*

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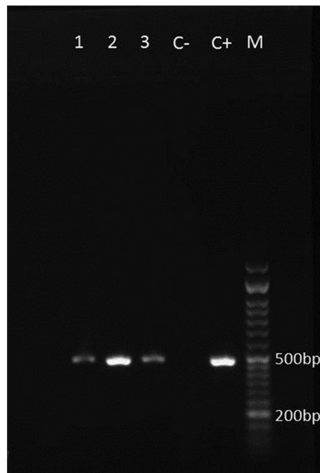

Figure 2: 1.5% Agarose gel electrophoresis of the polymerase chain reaction product of *Toxoplasma gondii.* M: 50‑bp DNA ladder. C+: Positive control, C−: Negative control, Lane 1–3 samples

have reported a prevalence of cattle sarcocytosis between 52% and 100%, worldwide.[27,28]

Previous studies in Iran revealed a high prevalence of cattle sarcocystosis.[9,29] For example, according to studies performed in Iran, 100% of cattle in Kerman and Ahvaz were found infected by *Sarcocystis* using a digestive method.[30,31]

The present study showed that mixed infection to *S. cruzi* and *S. hominis* is the most prevalent species followed by *S. cruzi*, in native cattle of Kashan, central Iran (83% to 84.9%). This is an important finding because *S. cruzi* causes acute systemic illness, poor growth, and occasionally abortion in cattle, and *S. hominis* is a human pathogen and possesses zoonotic importance. Humans can serve as definitive hosts, with intestinal sarcocystosis for *S. hominis*, through eating undercooked meat from beef.[8,9] This finding is in agreement with most of the previous studies about the prevalence and species identification of cattle sarcocystosis. In many countries, over 90% of adult cattle have been found infected with *S. cruzi*, and it is the most prevalent *Sarcocystis* species infecting cattle globally.[32] In previous literature concerned Iran, a high rate of *S. cruzi* infection had been reported in slaughtered cattle. Hajimohammadi *et al*. reported that 90% of slaughtered cattle in Yazd, Iran, were infected by *S. cruzi*, using the PCR‑RFLP method.[9] Another molecular analysis of *Sarcocystis* infections in cattle slaughtered in the northwest of Iran showed that 87.9% of isolates were identified as *S. cruzi*.[33] In Iran, the free access of stray dogs to pastures can contaminate the cattle with the infective stages of *S. cruzi*.

Similar to our findings, some recent molecular and histopathological investigations show a relatively high percentage of *S. hominis* infection in slaughtered cattle worldwide.[34-36] The prevalence of *S. hominis* in cattle had been reported between 6.2% and 97.4% in Europe, Asia, and South America.[10] In Iran, the results of studies on the prevalence of *S. hominis* in cattle are confusing and controversial. This may be due to the imported cattle from other regions and countries to Iran. The prevalence of *S. hominis* in slaughtered

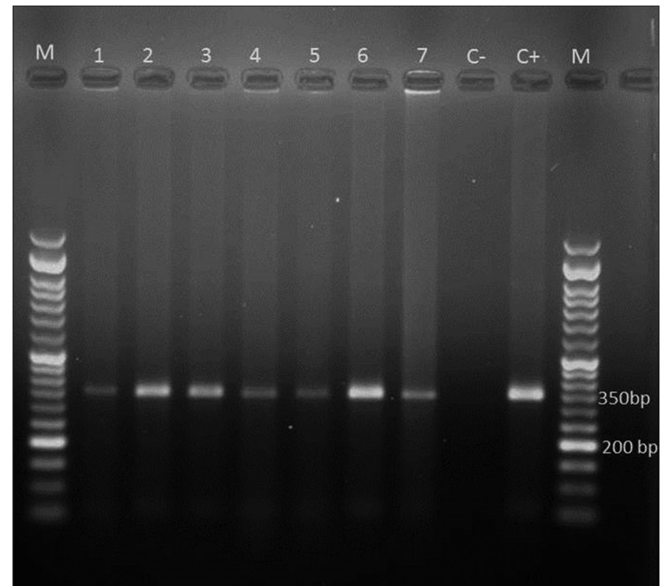

Figure 3: 1.5% Agarose gel electrophoresis of the polymerase chain reaction product of *Neospora caninum.* M: 50‑bp DNA ladder. C+: Positive control, C−: Negative control, Lane 1–7 Samples

cattle in Iran has been reported as high as 57.5% in Yazd, Central Iran, and 54.4% (mixed infection to *S. cruzi* and *S. hominis*) in Shiraz, southwest, to low as 1.03% in the northwest provinces of Iran.[9,33,37] Therefore, further studies are necessary to determine the accurate prevalence of *S. hominis* throughout Iran. This seems to be important due to the common traditional consumption of undercooked beef in Iran.

It is interesting to note that in the present study the corresponding 108 bp amplicon for *S. hirsuta* was not detected in any of the samples. To the best of our knowledge, the occurrence of *S. hirsuta* in Iranian native cattle is very rare and only one case report was published which showed that *S. hirsuta* was isolated from diaphragm of a 2‑year‑old slaughtered cattle in Shiraz, Iran.[38] It is well‑established that only cysts of *S. hirsuta* can become macroscopic in the tissue of cattle. There is no report about the prevalence or occurrence of *S. hirsuta* cyst in cattle using macroscopic or digestive methods in Iran.[30,31,33,37,38]

These finding points out that the environment is low contaminated with sporocyst of *S. hirsuta* disposal of the cat as the definitive host or due to the absence of the species in Iran. In contrast to stray dogs, cat populations are very low in farms, and they are mostly near the human houses in Iran. However, a previous study on industrial hamburgers prepared for sale in Kashan, showed 20.7% had co‑infection to *S. cruzi* and *S. hirsuta*[29]. This may be due to the imported beef from other countries for the production of industrial hamburgers in Iran.

In the present study, the positivity to *T. gondii* by conventional PCR was found to be 5.7% (5/53 muscle samples). Although toxoplasmosis is the most common protozoan zoonosis in humans and up to 50% of all human toxoplasmosis cases are foodborne infection,[39] cattle are usually considered as poor hosts for *T. gondii*.

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The seroprevalence of toxoplasmosis in cattle was estimated 9% worldwide, so cattle are not an important reservoir of *T. gondii*.[16] According to Dubey and Thulliez, cattle toxoplasmosis does not usually cause clinical symptoms, and there is high natural resistance to *T. gondii* in them.[40] A comparative study in Iran showed that cattle are relatively resistant and likely infected by *T. gondii* by six times less than sheep.[41]

The finding of the present study is similar to the findings of Azizi *et al*.,[41] Rahdar *et al*.,[42] and Lefkaditis *et al*.,[43] who reported a low prevalence of *T. gondii* in cattle. Another study from Isfahan and Chaharmahal va Bakhtiary provinces, central and south-west of Iran, showed no evidence (0%) of contamination with *T. gondii* in the 155 samples of cattle using PCR method.[44]

However, there are other studies that obtained high levels of *T. gondii* infection among cattle, which is in contradiction to these findings.[45,46] Therefore, it is recommended to avoid eating undercooked bovine meat products for preventing human infection.

Our results demonstrated that 23.9% of the samples test, contained *N. caninum* DNA that indicated a considerable prevalence of infection. To our knowledge, there has been no survey on neosporosis among beef or cattle meat products by molecular methods in Iran.

The first report of the presence of *N. caninum* antibodies in healthy and aborted dairy cattle in Iran was in 2004.[47] Since that time, many studies from different regions of Iran have been published, mostly on the seroprevalence of the parasite in different animals or aborted bovine fetuses.[19] However, there is little information about the molecular frequency of *N. caninum* related to aborted bovine fetuses in Iran. Sadrebazzaz *et al*. showed that a total 33% of bovine fetuses were considered to be infected with *N. caninum* by PCR technique.[48] Another study in Tabriz, northwest Iran, diagnosed *N. caninum* in 42.8% of brain samples of an aborted fetus by PCR method.[49] These findings indicated that *N. caninum* is an important cause of bovine abortion in Iran.

PCR positivity rate of *N. caninum* in our study was less than prevalence (36.5%) amongst beef cattle in Phayao, Thailand,[23] and was higher than of prevalence in cattle (12.36%) in Wasit province, Iraq,[50] and 22% of cattle in North Africa.[51]

In this study, no association between the age and sex of animals and infection by *N. caninum* was observed which is consistent with the other similar studies.[23,50,51] In contrast, in other seroprevalence studies, a significant relationship between the age of animal and seropositivity showed that confirmed older animals are more likely to be infected by acquiring the oocyst of *N. caninum* from the environment through a lifetime.[19,23,43]

Due to the high prevalence of *N. caninum* in native cattle of Kashan, prevention of contamination of food and water supplies, bedding, and pastures with canine feces are necessary to its control.

Conclusion

It is concluded that PCR is a suitable method to diagnose *Sarcocystis* sp., *T. gondii*, and *N. caninum* infections in cattle simultaneously. This study revealed a high prevalence of infection to *N. caninum* and *S. cruzi* or mixed infection of *S. cruzi* with *S. hominis* among slaughtered cattle in Kashan, Iran. To avoid contamination of water, feed, and bedding with an infectious stage of *Sarcocystis* and *N. caninum*, prevention measures such as keeping away dogs from cattle grazing by fencing techniques should be done.

Although in this study, the prevalence rate of *T. gondii* was low and cannot be considered as a major zoonosis, it should be strongly recommended to avoid eating raw or under-cooked bovine meat products to prevent human infection to *T. gondii* and *S. hominis*.

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Conflicts of interest

There are no conflicts of interest.

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