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Molecular diagnosis of *Sarcocystis hominis* and *Sarcocystis cruzi* in slaughtered cattle in Tabriz, Iran using PCR- RFLP with new digestion patterns

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Abstract

Sarcocystis species are intracellular protozoan parasites that cause cysts in the muscles of animals throughout the world. So far, more than 200 *Sarcocystis* species with different life cycles and pathogenicity have been identified in different hosts, and some of these species have zoonotic importance. Seven species in cattle are known to form macroscopic and microscopic cysts. Of which, *S. hominis* and *S. heydorni*, are zoonotic species and *S. cruzi* is the most pathogenic species in cattle. This study aimed to identify the important species of *Sarcocystis* in thirty cows slaughtered in Tabriz slaughterhouse by sampling the heart, diaphragm, intercostal and triceps muscles. Although no macroscopic cysts were found, prevalence of microscopic cysts were 86.6%. The results showed 926 bp and 937 bp fragments for *Sarcocystis hominis* and *Sarcocystis cruzi*, respectively. The *BfaI* endonuclease was used to digest the PCR products. According to the PCR-RFLP results, the rate of single infection with *S. hominis* and *S. cruzi* was 16.7% and 26.7%, respectively, and co-infection was 43.2 %. *S. cruzi* was showed ~ 400bp, 300 bp, and ~ 237 bp fragments and *S. hominis* was showed one ~600bp with two overlapped ~163 bp (2x). In the present study, new digestion patterns were observed compared to previously reported in Iran with the same restriction enzyme. These results suggest the presence of heterozygosity in *S.cruzi* and *S.hominis* in different regions of Iran.

Introduction

Sarcocystis sp. is a cyst-forming coccidial parasite with a two-host life cycle including carnivores as definitive hosts, and herbivores or omnivores as intermediate hosts (1). More than one species of *Sarcocystis* may exist in every intermediate and definitive host (2). Ultrastructural studies and molecular biology procedures (PCR and sequencing) were used to distinguish and differentiate *Sarcocystis* species in intermediate hosts (3, 4). Formerly, *Sarcocystis* were determined by the structure of the cyst wall, which typically

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originates in the intermediate host's striated muscles. Nowadays, examining of DNA sequences helps to identify *Sarcocystis* species. Even within the same genus, variable regions of the 18S rRNA gene are compelling targets for identifying and characterizing distinct species (5). So far, it has been found that 18SrRNA sequences show the lowest interspecies homology for *Sarcocystis* compared to intraspecies comparisons (6-10).

RFLP analysis is a valid and reliable diagnostic tool for *Sarcocystis* spp. This method is based on the fragmentation of the nucleic acid obtained from the PCR reaction. To perform the RFLP test, restriction enzymes must be used to digest nucleic acids. After digestion, researchers obtain a precise pattern of nucleic acid fragments. Sequence diversity is defined by comparisons between digestion results. This method is described as a low-cost method, with simple use and excellent measurement sensitivity (10).

So far, seven species of *Sarcocystis* in cattle have been reported to have different pathogenicity. *Sarcocystis cruzi* is recognized as the most pathogenic species, that causes abortion, low milk yield, poor body growth, and death in cattle, and *S.hominis* is the most important zoonotic species from cattle, and transmitted to humans by eating raw or undercooked beef (11). Therefore, this study aimed to detect *S.cruzi* as important pathogenic species in cattle and *S. hominis* infection rate as zoonotic species in beef for human consumption in Tabriz, Iran.

Material and methods

Sampling

This study was conducted on 30 samples randomly taken from the heart, diaphragm, intercostal and triceps muscles of the slaughtered cattle from the Tabriz slaughterhouse, Iran. All animals in this study appeared healthy before slaughter. To diagnose macroscopic sarcocysts, eye examination was used, and for microscopic cysts, peptic digestion and molecular methods were used.

Peptic digestion method

For the presence of *Sarcocystis* macrocysts, the sampled muscles were thoroughly examined, and after that, the peptic digestion method was applied as formerly described (12). Briefly, fifty grams of grind muscles were incubated in 100 mL of acid-pepsin for 30 minutes at 37°C as a digestion medium (10ml HCl 37%+2.5 gr pepsin). Then, 200 ml of PBS was added to the solution to inactivate the acid. The digested material was filtered into a tube through a fine mesh filter, centrifuged at 1500 ×g for 10 minutes, and the sediment suspended in 0.5 ml of distilled water. The suspension was then examined under a light microscope at 400× magnification for *Sarcocystis* bradyzoites. In addition, more drops of the same solution were applied to the glass slides, fixed, and stained with 1% Giemsa stain, and re-examined.

DNA extraction and PCR reaction

Genomic DNA was isolated using the phenol-chloroform technique, then stored at -20 °C. The polymerase chain reaction was performed to amplify the 18S ribosomal RNA gene of *S.hominis* and *S.cruzi* using forward primer (5'-CGT GGT AAT TCT ATG GCT AAT ACA-3') and reverse primer (5'-TTT ATG GTT AAG ACT ACG ACG GTA-3') as previously described (10). The expected PCR products were 926 bp for *S. hominis* and 937 bp for *S. cruzi*.

The PCR reaction was performed by a 25 µL reaction mix comprising genomic DNA (100 ng), 1µL of each primer (10 µM), 12.5 µL of master mix PCR (Amplicon, Denmark), and distilled water. In a Eurofins thermal cycler, the reaction was carried out (Eurofins, Germany). PCR program set upped as a 5-minute denaturation stage at 94°C was follow out via 30 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C and a 5-minute final elongation step at 72°C.

RFLP analysis

The PCR products were independently digested by *BfaI* restriction endonuclease (Thermo SCIENTIFIC, Canada) to identify *S. cruzi* and *S. hominis*, according to the manufacturer's instructions. Briefly, a total volume of 30 µl was

prepared from the digestion reaction containing 8 µl of PCR products, 1 µL (1 U) of *BfaI* restriction enzymes, 3 µL of 10x enzyme buffer, and 18 µL of distilled water. For enzyme activation, reaction tubes were incubated for 16 hours at 37 ° C, then 20 minutes at 65 °C for inactivation. The digested PCR products were simultaneously loaded with a 50-1500 bp DNA ladder (SinaClon, Iran) on a DNA safe-stained 1.2% agarose gel (SinaClon, Iran) and imaged.

Results

Macroscopic and Microscopic Results

The presence of microscopic Sarcocysts in cattle was found in 26 out of 30 samples (86.66%) by the digestion method. The bradyzoites of *Sarcocystis* in beef are illustrated in Figure 1. No macroscopic cysts were observed.

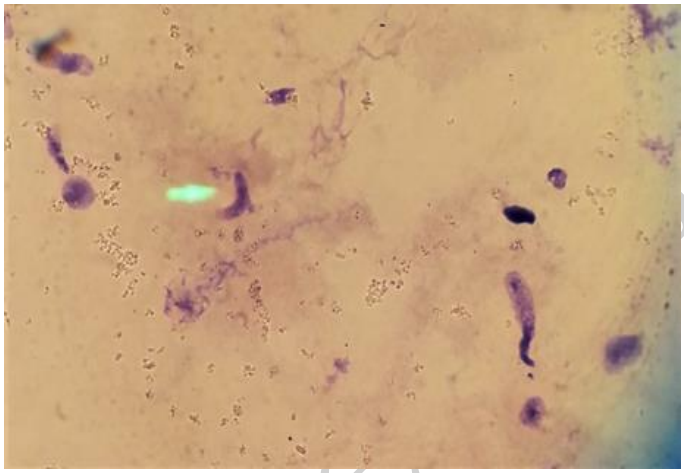

Fig. 1. Bradyzoites of *Sarcocystis* in Giemsa staining by digestion method (1000x).

PCR results

In molecular study, out of 30 samples, 26 samples (86.6%) were positive. The results of 1.2% gel agarose electrophoresis showed a 926 bp and a 937 bp of PCR products for *S. hominis* and *S. cruzi,* respectively (Figure 2). Four samples didn't have any contamination either in the microscopic or molecular study.

RFLP analysis

The *BfaI* endonuclease was used to digest 8 µl of PCR products. Digestions were electrophoresed on %2 agarose gels with a voltage of 60 volts (120 minutes). According to the PCR-RFLP results, *S. cruzi* was offered ˷ 400 bp and ˷ 300bp, and also ˷ 237bp fragments. Also, *S. hominis* was showed one ˷600bp with two overlapped ˷163 bp fragments (Figures 3, 4).

All positive samples of *S. hominis* with each other and all positive samples of *S. cruzi* with each other showed homozygosis in the 18S rRNA gene because two and three restriction enzyme sites were seen in the genes of the two species in all samples, respectively. The rate of single infection with *S. hominis* and *S. cruzi* was about 16.7% and 26.7%, respectively, and coinfection was 43.2%. In total, the infection rate of *S.cruzi* was 69.9% and *S. hominis* was 59.9%. We found new digestion patterns in comparison to previously reported fragments with the same restriction enzyme in Iran.

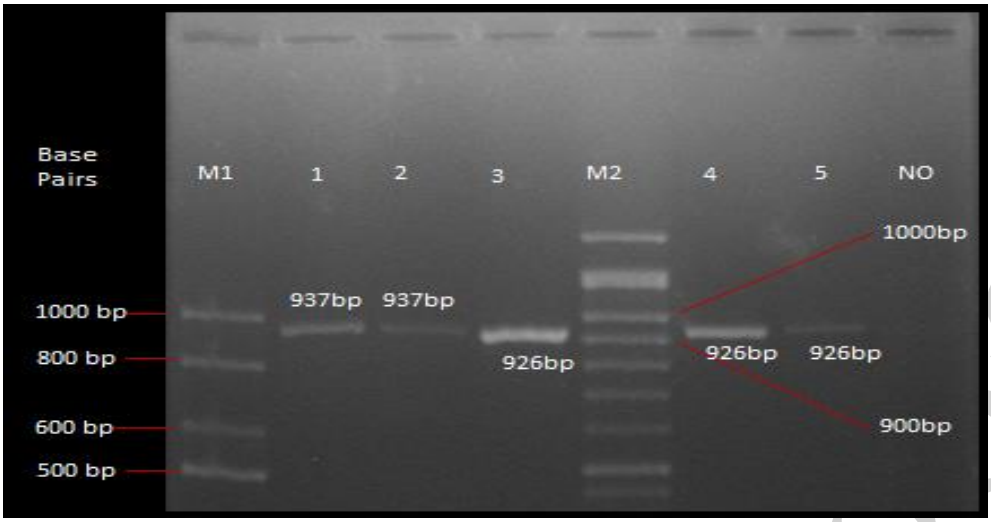

Fig. 2. Gel electrophoresis of amplification products (PCR) obtained from amplified 18S ribosomal RNA gene on 1.2% agarose gel**.** M1: DNA ladder 100 bp (Jena bioscience, Germany); lanes 1&2: PCR products of *S. cruzi*; M2: DNA ladder 50 bp, low range (SinaClon, Iran); lanes 3-5: PCR products of *S. hominis.*NO: Negative control

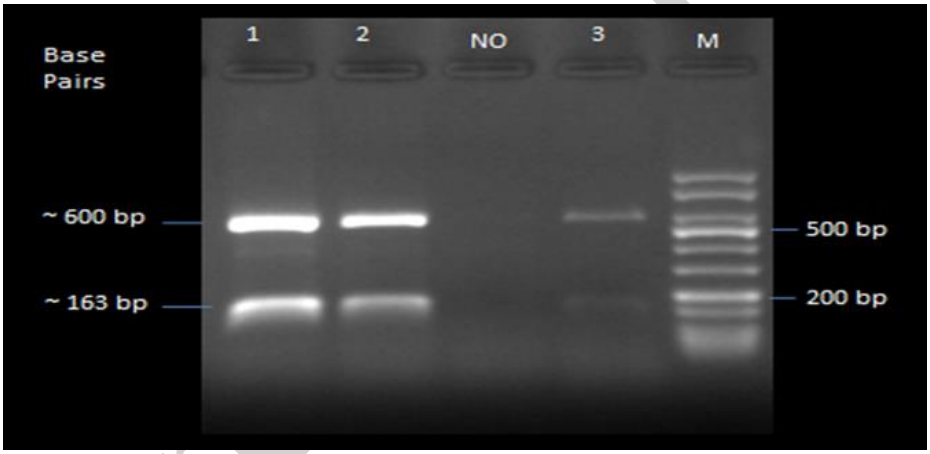

Fig. 3. Gel electrophoresis of *S. hominis* digestion products by *BfaI* endonuclease on 2% agarose gel. Lanes 1-3: RFLP results using *BfaI* endonuclease showed ˷ 600bp and ˷ 163bp (2x) bands on *S. hominis* PCR products with 926bp. M: DNA ladder 50-1000, low range (SinaClon, Iran). NO: Negative control.

Discussion

In addition to the zoonotic importance of *S. hominis*, eating beef infected with *S. cruzi* can also cause digestive symptoms such as nausea and diarrhea in humans. Therefore, this research was conducted to investigate the level of contamination with these species in beef in Tabriz. To characterize *S. cruzi* and *S. hominis* in slaughtered cattle, the current study used a restriction fragment length polymorphism (RFLP) technique. It showed the distinction between these species, using only a restriction enzyme (*BfaI*) at 18S rRNA gene.

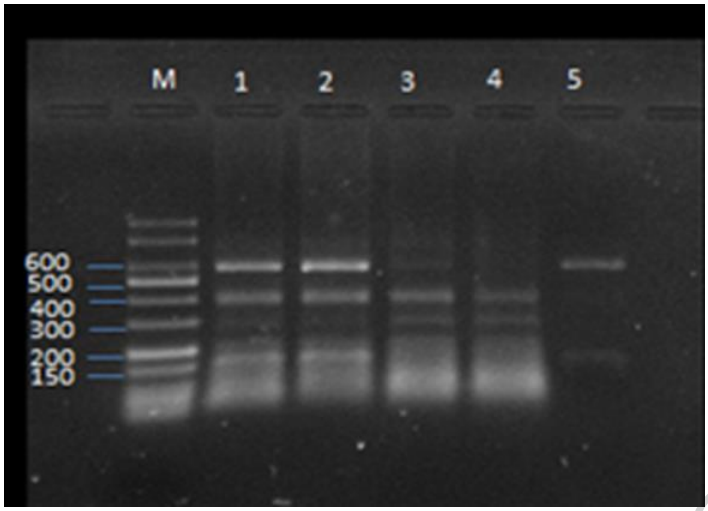

Fig. 4. Gel electrophoresis of amplification products (PCR) by *Bfa*I endonuclease on 2% agarose gel. Lanes 1-2: RFLP products using *BfaI* endonuclease showed coinfection of *S. cruzi* and *S. hominis*. Lanes 3-4: RFLP products using *BfaI* endonuclease showed ~ 400bp, ~ 300 bp and ~ 237bp on *S. cruzi* with 937bp PCR products. Lane 5: RFLP results using *BfaI* endonuclease showed ~ 600bp and ~ 163bp (2x) bands on *S. hominis* PCR products with 926bp. M: DNA ladder 50-1000, low range (SinaClon, Iran).

Many practical tools for the detection and differentiation of *Sarcocystis* spp. exists and allows scientists to conduct epidemiological research. Morphological assessment of *Sarcocystis*, especially the structure of the cyst wall and sporocysts is used to distinguish different species, but the available molecular methods are able to identify morphologically similar cysts or belonging to other species in each host (13, 14). The application of PCR-RFLP technique in the routine identification of *Sarcocystis* species has been proven as a rapid and more cost-effective technique, gives the same result as detailed morphological studies or DNA sequence-based identifications (10, 15).

RFLP with *BfaI* in this study showed a different enzyme digestion pattern with previously reported in Iran in slaughtered cattle. Haji Mohammadi et al. (2014) reported the restriction fragments of 376 bp and 397 bp detected *S. hominis* and fragments of 184bp, 371bp and 382 bp detected *S. cruzi*, after RFLP with *BfaI* in Yazd, Iran (16). Also, Dalir Ghaffari et al. (2022) reported the restriction fragments of 560/70/50 bp detected *S. hominis* and fragments of 365/195/70/50 bp detected *S. cruzi*, after RFLP with *BfaI* in west of Tehran, Iran (17). These results suggest the presence of heterozygosity in *S.cruzi* and *S.hominis* in different regions of Iran. As in a recent study in Northwest Iran by Sarafaraz et al. (2020) the heterogeneity analysis of *S. cruzi* 18S-rRNA sequences has indicated the genetic diversity of *S. cruzi* isolates including 16 haplotypes (18). The prevalence of *S. hominis* and *S. cruzi* in cattle in this study was consistent with previous reports in other countries in that *S. cruzi* was the most common species, followed by *S. hominis* (2, 19- 21). Also, this study like previous showed that *Sarcocystis* infection is very common in cattle in Iran, and the dominant species is *S. cruzi* (16, 18, 19, 22).

Conclusion

In the present study, the rate of single infection with *S. hominis* and *S. cruzi* was 16.7% and 26.7%, and coinfection, was 43.2 %. *BfaI* endonuclease could be detected *S. cruzi* and *S. hominis*. But, new digestion RFLP patterns were observed compared to previously reported. Finally, it was concluded that RFLP- PCR technique is strong and useful molecular tool can detect heterogenicity in *Sarcocystis* species successfully.

Acknowledgments

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Ethical approval

This study was conducted following the guidelines and standards of the Animal Research Ethics Committee at the University of Tabriz.

Conflict of interest

There is no conflict of interest in conducting this research.

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