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Original Article

The Infestation of Supplied Mincemeat to *Sarcocystis* Species and Their Polymorphism Using PCR-SSCP Method

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Abstract

Background: *Sarcocystis* spp. is zoonotic parasitic pathogen endangering the safety of meat and derived meat products. This study was carried on for determination of presence of *Sarcocystis* species in mincemeat in Tabriz (Iran) using single-stranded conformation polymorphism (SSCP) method.

Methods: Thirty samples of mixed ground meat were collected from butchers and DNA was extracted. The 18S rRNA gene fragment of *S. cruzi* and *S. hominis* species in the extracted DNA amplified by PCR using one pair specific primers, which resulted in 937 bp length fragments in parasite-positive samples. The results showed that 11 samples out of 30 (36%) were positive for *S. cruzi* and *S. hominis* species. The SSCP technique products of gene indicated 4 SSCP patterns (A, B, C and D) among study samples on 18S rRNA gene of *S. cruzi* and *S. hominis*.

Results: Contamination with *Sarcocystis* species in minced meat sold in Tabriz City in Iran is relatively high, which is of particular importance from a health point of view. This study was currently by SSCP technique on minced meat for the first time, based on the primers in the 18S rRNA gene locus, there are polymorphisms in *S. cruzi* and *S. hominis* species.

Conclusion: Due to the relatively high level of *Sarcocystis* contamination in minced meats in Tabriz, the necessary hygienic measures should be must implemented. Also, there was polymorphism in *S. cruzi* and *S. homonis*.

Introduction

Sarcocystosis is a parasitic zoonotic disease caused by species of *Sarcocystis*; intracellular protozoan parasite in the phylum Apicomplexa and family Sarcocystidae. *Sarcocystis* has a required two-host life cycle based on a prey-predator (intermediate-definitive) host relationship (1). Cats and dogs are recognized as definitive hosts, while cattle

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(carnivores), pigs, horses, sheep, goats, birds, rodents, camels, wild animals, reptiles and humans are known intermediate hosts in which *Sarcocyst* cysts develop in skeletal muscles. Humans are the definitive hosts of two *Sarcocystis* species: *S. hominis* (bovihominis) and *S. suihominis* (2). *S. cruzi* is transmitted between canines (as definitive host) and cattle (as intermediate host), and does not cause infection in humans.

In humans, eating raw or undercooked meat containing mature *Sarcocystis* has caused intestinal sarcocystosis in infected individuals, which manifest as diarrhea, bloat, dyspnea, tachycardia and loss of appetite (1). Sarcosystosis is most often asymptomatic in definitive hosts, but mild self-limiting diarrhea has been reported Infection is characterized by cyst formation in muscles tissues in the intermediate host (muscular sarcocystosis) or by colonization of the intestinal lining in definitive host (intestinal sarcocystosis) in the.

In pathological methods, gross examination, impression smears, and digestion methods are the common methods for diagnosis of the disease and indirect fluorescent antibody test (IFAT), and Enzyme-linked immunosorbent assay (ELISA) are used as serological methods for diagnosis of the disease (3). Since these methods take time or are non-sensitive enough to identify *Sarcocystis* species as well as other methods, DNA-based analysis methods help to identify all species (4).Different regions of the 18S rRNA gene provide useful targets for the detection and characterization of different species, even within the same genus (5).The single-stranded conformation polymorphism (SSCP) procedure involves PCR amplification of the target fragment, denaturation of the amplified PCR product with heat and formamide, and electrophoresis on a nondenaturing polyacrylamide gel. The SSCP method is based on the observation that under non-denaturing conditions, single stranded DNA (ssDNA) fragments break into different conformations stabilized by their primary sequence, and the formation is controlled by intramolecular interactions (6). Therefore, even a single opening can cause a change in conformation, which can be observed by changing the movement of the single-stranded DNA molecule in SSCP. In different parts of Iran sarcocystosis has been investigated in different food animals, which indicate the infection rate in range between 3.5 and 100 % (7-10).

Minced meat is consumed in large amount in Iran. Therefore, its health is very important in public health. Determining the presence of *Sarcocystis* in meat products intended for human consumption is important given the economic loss to the meat industry and its public health impact due to the high prevalence of this parasite. We aimed to detect the contamination of supplied mincemeat with Sarcocysts species and its polymorphism in Tabriz (Iran) by PCR-SSCP method.

Materials and Methods

The study was conducted in Tabriz (northwest Iran). During the fall of 2023 the samples were collected randomly from butchers. One hundred grams of mixed ground meat was collected from 30 butchers and immediately transferred to the parasitology laboratory of the Faculty of Veterinary Medicine of University of Tabriz. The samples were then numbered and frozen at -20 °C until testing.

DNA extraction from ground meat using phenol, chloroform, and isoamyl alcohol

DNA was extracted by Campos and Gilbert with some modification (11). Briefly, 50 mg of ground meat was with a tissue grinder into 1.5 mL microtubes, then 200 µl distilled water was added and vortex. Then the samples three times freeze and thawed at -80°C. 500 µl sodium hydroxide (pH=12, M=1.0) with sodium dodecyl sulfate (1%) added, vortex and keep at -80 °C for 20 min. 500 µl of Phenol, chloroform, isoamyl alcohol (25:24:1) were added

and centrifuged at 10,000 g. for 5 min, which resulted in 3 phases.

The supernatant was transferred to new microtube and added 500 µl of chloroform, vortexed and centrifuged at 10,000 g. for 5 min resulted in 2 phases. The supernatant carefully removed and transferred it to a 1.5 mL new microtube. Isopropyl alcohol added at a ratio of 0.6-1 and sodium acetate at a ratio of 0.1 of the total volume of the solution. The samples centrifuged at 10,000 g. for 10 min to pelleted DNA. DNA pelleted washed with 500-1000 µl of ethanol 85% and then 50 µl of distilled water added and stored at -20 °C.

Agarose Gel Electrophoresis

The quality and quantity of extracted DNA was determined by 0.8% Agarose gel with the standard DNA (DNA ladder, Sinaclon Co.) for an hour at 90 volts. Then PCR was run with specific primers and PCR products visualize on the 1.5% Agarose gel.

Polymerase chain reaction (PCR) condition and program

PCR was run by thermal cycler (MWGAG Biotech, Germany) with 25 µl total reaction, using 12.5 µl Master Mix (Ampliqon, Denmark), 5 µl of extracted DNA (≃ 0.5-1 µg), 1 µl (1µM) of forward and 1 µl reverse primer, 5.5 µl distilled water. Primers for amplification of 18S ribosomal RNA gene were used according to sequences from Yang et al as well as sarF 5'-CGT GGT AAT TCT ATG GCT AAT ACA-3' and sarR 5'-TTT ATG GTT AAG ACT ACG ACG GTA-3' (GenBank: MG787082) (12).

Thermal cycler program was as follow; a 94 °C Initial denaturation for 3 min (1x), then 30 cycle a 94 °C Denaturation for 60 s, 54 °C Annealing for 60 s, 72 °C Extension for 60 s, and 72 °C a final extension step (1x) for 5min.

Single Strand Conformation Polymorphism (SSCP) gel

SSCP gel (10%) in TBE 1X buffer was prepared as follow; the first a polyacrylamide gel 30% (14.5 g acrylamide and 0.5 g bis-acrylamide) were prepared in a 50 mL Falcon tube with distilled water. Then an acrylamide gel with 36 ml volume (according to the glass volume) was made as follow; 8 ml polyacrylamide gel 30%, 23 ml distilled water, 3.5 ml TBE 10X, 1 mL Glycerol, 500 µl ammonium persulfate (0.1%, v/w), and finally 50 µl TEMED.

Before loading the samples on SSCP gel, the PCR products were single stranded as well as; 6 µl of the PCR product was mixed with 4 µl of the sample buffer (50 µl of dye 6X and 940 µl of formamid were mixed with 10 µl of NaOH (1.5%) and heated at 95 °C for 10 min and immediately transferred on ice. Then the samples loaded on the SSCP gel and electrophoresed at 120 volts for 6 hours.

SSCP gel staining with silver nitrate

The staining steps were as follows; the gel was placed in 250 ml of fixing solution (6 ml of glacial acetic acid with 50 ml of ethanol 96% and 450 ml of distilled water) and shaken in the solution for the first 10 min and the second 5 min. Then, the gel was rinsed with distilled water twice for 20 second and placed in the staining solution (silver nitrate 0.1%) and shaken for 20 min. This step was performed in the dark. The gel was rinsed twice with distilled water for 20 s. The gel was placed in the developer solution (15 g of NaOH, 1 ml formaldehyde 17%-, and 1000-ml distilled water) and shaken for about 20 min until the appearance of bands. Finally, the gel was placed in the stop solution (3% acetic acid) for 15 min.

Results

DNA of all collected meat samples (30 samples) from slaughterers in Tabriz-Iran was extracted and some samples randomly run on the 0.8 % agarose gel for evaluation of quality and quantity of extracted DNA (Fig. 1).

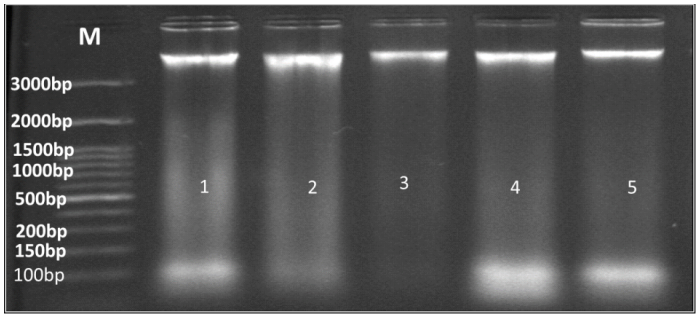

Fig. 1: Electrophoresis of extracted DNAs on 0.8% agarose gel. M: DNA ladder (Jena bioscience, Germany3000 bp); lanes 1-5: the extracted DNAs

Amplification of the 18S rRNA gene of cruzi and hominis species

The of 18 sRNA gene fragment of *S. cruzi* and *S. hominis* species in the extracted DNA through PCR using one pair specific primers were amplified, which resulted in 937 bp length fragments in parasite-positive samples. No amplification occurred in samples that lacked these species.

The PCR products were electrophoresed on 1.5% agarose gel. The results showed that 11 samples out of 30 (36%) were positive for *S. cruzi* and *S. hominis* species (Figs. 2 and 3).

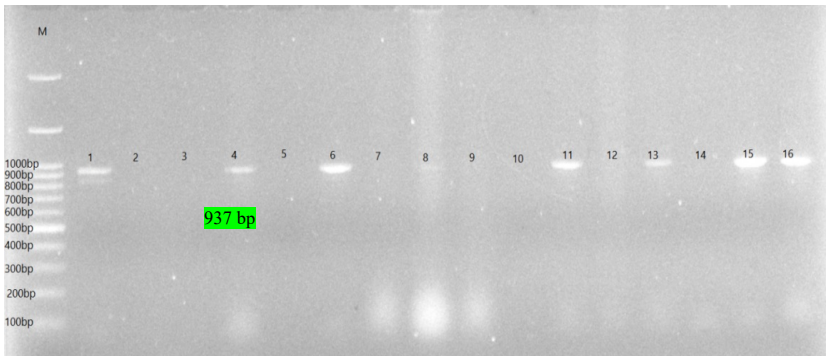

Fig. 2: PCR products electrophoresis on 1.5% agarose gel. M: DNA ladder (Jena bioscience, Germany,3000 bp), 50-1000 bp; lanes 1, 4, 6, 8, 11, 13, 15, and 16 indicate the 18S rRNA gene amplification using the specific primers

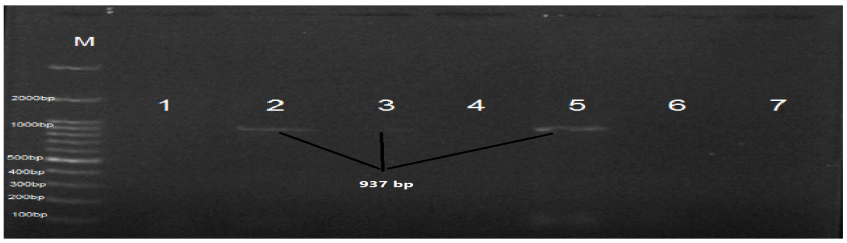

Fig. 3: Electrophoresis of other PCR samples on 1.5% agarose gel. M: DNA ladder (Jena bioscience, Germany), 50-1000 bp; lanes 2, 3, and 5 indicate the 18S rRNA gene amplification which were positive for S. *cruzi and S. hominis parasites.7: negative control*

The results of the SSCP pattern

SSCP pattern in the *S. hominis* and *S. cruzi* gene location is showed in Fig. 4. The SSCP technique showed that in this gene location, 19 samples were showed monomorphic patterns, and 4 different patterns (A, B, C and D) were indicated in 11 samples. Six out of eleven were showed A pattern; 2 sample pattern B; 2 sample pattern C and 1 sample pattern D, for the *18S rRNA* gene of *S. cruzi* and *S. hominis* (Fig. 4). In the studied samples, single-strand conformation polymorphism as a powerful technique could identify single nucleotide mutations and can be used in a large number of samples, from microorganisms to humans.

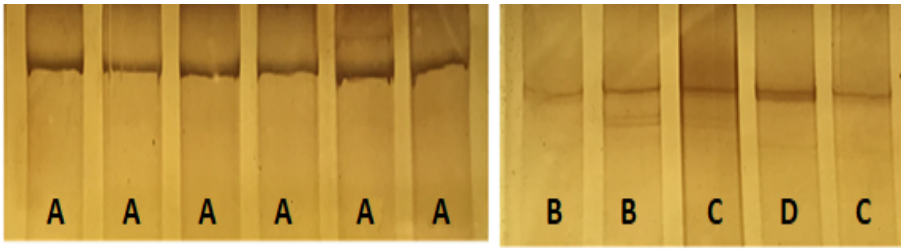

Fig. 4: The SSCP patterns of PCR products from amplified region of 18S rRNA of *S. hominis* and *S. cruzi*

Discussion

Eleven of the 30 samples collected in this study were positive for *S. cruzi* and *S. hominis*. Sarcocystosis was diagnosed in 36% of minced meat in Tabriz (Iran). This finding should be considered by health officials, because *S. hominis* have zoonotic importance and eating the meat infected by mentioned *Sarcocystis* cause digestive symptoms such as nausea and diarrhea in humans. The importance of this issue increases when we know that most of the minced meats are used to prepare some kind of food in Iran, which receive the least amount of heat when cooking.

Several studies have showed various percentage rates for *Sarcocystis* prevalence in the raw meat, Hamburger and other products that reach human consumption. These reports based on molecular detection of the agent and unlike the reports that rely on microscopic observations, which have a high probability of error, they are valid.

The first molecular identification of *S. cruzi* isolated in Iran was performed by Kalantari et al. PCR and partial sequence analysis of the *18S rRNA* gene were used to identify *Sarcocystis* species in commercial root samples (13). Of 25 commercial hamburger samples, 17 samples showed a PCR product of approximately 900 bp that could identify *Sarcocyst* Spp. The suthors identified restriction fragment 376 bp and 397 bp *S. hominis* or *S. hirsuta* and fragments of 184 bp, 371 bp and 382 bp detected *S. cruzi* (14). Another study showed a contamination rate of 29% in 200 hamburgers in Kashan, using PCR-RFLP method. Overall, 74% of the samples were infected by *S. cruzi*, of which canids acted as definitive host, while 20% were infected with both *S. cruzi* and *S. hirsuta* (felids as final hosts) and 3.5% revealed both infection of *S. cruzi* and *S. homminis* (man as final hosts) (15).

Bradyzoites of *Sarcocystis* were detected in 46 of 80 (57.6%) hamburgers samples in Hamedan. Positive specimens were included as 46 (57.6%) and 30 (37.5%) by digestion and molecular method, respectively. Differences between two studied (digestion and molecular) methods was statistically significant (16).

Comparing the levels of contamination reported in the above research with the current study shows that the level of contamination of ground beef in this research is consistent with

the level of contamination demonstrated in other parts of the country.

In a study similar to our study, 17 samples were positive for a 900bp product of *Sarcocystis* among 25 commercial hamburger samples. After RFLP with BfaI, the restriction fragments of 376 bp and 397 bp detected *S. hominis* or *S. hirsuta* and fragments of 184 bp, 371 bp and 382 (17).

PCR-SSCP is a new and widely used method for mutation detection in basic and applied biological sciences. Therefore, PCR-SSCP is still considered a method not only to study potential sequence changes, but also to identify new mutations (18).

A polymorphism is a gene rearrangement or mutation that allows different parts of a gene to have different evolutionary histories. The rate of sequence evolution is equal to the proportion of loci that change over a given period of time (the number of substitutions per locus over a billion years). Growth rates may vary from protein to protein. There are different types of nucleotide substitutions depending on their location and function in the genome. On the other hand, the detection of gene polymorphisms using PCR-based genetic markers, including SSCP, is one of the most reliable methods for detecting mutations.

Conclusion

Contamination with *Sarcocystis* species in minced meat sold in Tabriz is relatively high, which is of particular importance from a health point of view and it was determined for the first time that, based on the primers in the 18S rRNA gene locus, there is polymorphisms in *S. cruzi* and *S. hominis* species.

Acknowledgements

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Conflict of Interest

All authors declare that there is no conflict of interest.

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