Int J Enteric Pathog. 2014 November; 2(4): e21139. DOI: 10.17795/ijep21139

Published online 2014 November 8. **Research Article**

Molecular Method Development to Identify Foodborne *Sarcocystishominis* in Raw Beef Commercial Hamburger

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Received: June 13, 2014; **Revised:** July 21, 2014; **Accepted:** August 9, 2014

Background: *Sarcocystisspp*. is zoonotic parasitic pathogen endangering safety of meat and derived meat products such as hamburgers which is among the most popular fast foods worldwide.
Objectives: The current study aimed to design a protocol for molecular identification of *Sarcocystis hominis* in commercial hamburgers using PCR-RFLP with target of *18S rRNA*.
Materials and Methods: A total of 25 raw commercial hamburger samples were randomly collected from supermarkets of Yazd city, Iran. Five mm slices from different parts of each sample were selected, well mixed, and then preserved in ethanol 70% at -20°C for the next steps. The genomic DNA was extracted using salting out method. Detection and identification of *Sarcocystis* isolates were performed using PCR-RFLP. The *18s rRNA* gene sequence was mined from GenBank and the specific primer pair was designed using Primer3 software. Restriction fragment length polymorphims (RFLP) analysis was performed using *BfaI* and *RsaI* restriction enzymes. The digestion was analyzed, using agarose gel electrophoresis alongside 100base pair DNA ladder.
Results: Among 25 commercial hamburger samples, 17 samples showed a PCR product around 900 bp which could detect Sarcocyst *Spp*. 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 bp detected *S. cruzi*. After RFLP with *RsaI*, the restriction fragments of 376 bp and 557 bp detected *S. hirsuta* and fragment of 926 bp, without any digestion, detected *S. hominis*. For verification, each species detected in samples was randomly selected and sent for sequencing and the results were analyzed with BLAST.
Conclusions: In conclusion, the current study developed a practical technique to detect the prevalence of *S. hominis* in meat products such as hamburgers.

Keywords:Sarcocystisspp.; Food borne Diseases; Molecular Diagnostic Techniques

1. Background

Sarcocystisspp. as the obligatory intracellular two-host parasites belong to the family of Sarcocystidae and phylum of Apicomplexa (1, 2). Among the numerous species, just *S. hominis* and *S. suihominis* can infect humans, which cattle and pigs are their intermediate hosts, respectively. Intestinal infection in human may occur by consumption of undercooked beef or pork containing the larvae stage named sarcocyst (3-5). The clinical signs are digestive system disorders including nausea, vomiting, stomachache and diarrhea (3, 4). *Sarcocystis spp*. is considered as one of the important opportunistic agent in acquired immune deficiency syndrome (AIDS) patients (6). *Sarcocystis spp*. is zoonotic parasitic pathogens endangering safety of meat and derived meat products such as hamburgers which are among the most popular fast foods worldwide. Each year, about five billion hamburgers are consumed just in the USA (7). Although, there is no exact information regarding per capita of meat products in Iran, it is estimated that the annual consumption of hamburgers is considerable in this country. As beef is the main ingredient of hamburgers in Iran, the occurrence of *Sarcocystis spp*. in this product should be considered. The routine detection of these parasites includes pathological and serological methods. Gross examination, impression smears, and digestion methods are the usual methods in his to pathological methods and indirect fluorescent antibody test (IFAT), and Enzyme-linked immunosorbent assay (ELISA) are used in serological methods (8). As, these methods are either time-consuming or non-sensitive enough to identify *Sarcocystis* species in some methods with similar morphology, therefore, the techniques based on DNA analysis will be useful to identify any organisms (9), also

Sarcocystis species. The variable regions of the *18S rRNA* gene provide useful targets to identify and characterize different species, even from the same genus (10). It is found that such sequences for *Sarcocystis* show low interspecific homology relative to intraspecific comparisons.

2. Objectives

As *S. hominis* is the most important species transmitted by cattle in Iran, the current study aimed to design a protocol for molecular identification of *Sarcocystis hominis* in commercial hamburgers using PCR-RFLP targeting*18Sr-RNA*.

3. Materials and Methods

3.1. Sampling

A total of 25 raw frozen commercial hamburger samples with different meat content, from some factories were randomly purchased from supermarkets of Yazd city, Iran .In the laboratory, all samples were cut into 5 mm slices and examined carefully by naked eye for the presence of macro cysts of *Sarcocystis*. Five slices from different parts of each hamburger sample were selected and well mixed, and then preserved in ethanol 70% at -20°C for the next steps.

3.2. DNA Extraction

The genomic DNA was extracted using salting out method (11). Approximately 30mg of each sample was suspended in 900bμL NET (NaCl, 50 mM; EDTA pH 8, 25 mM, Tris-HCl pH 7.8, 50 mM) buffer, with 10 μL proteinase K (20 mg/mL, Fermentas, EO049), and Sodium dodecyl sulfate (SDS) with the end concentration of 1% in order to lysis the sample, followed by rapid mixing and incubation at 56 °C for an overnight. The purification of DNA was performed by adding 250 μL 6M NaCl. After centrifugation, the supernatant was transferred to a new sterile 1.5 mL micro tube. The DNA precipitation was performed by adding a double volume of chilled absolute ethanol. After washing with ethanol 70%, the DNA pellet was dried and re-suspended in 100μL of sterile double distilled water and stored at -20°C till next steps.

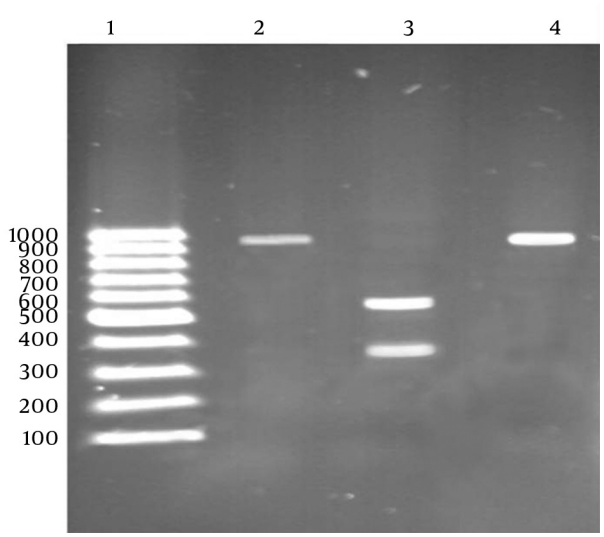
3.3. Detection and Identification

Detection and identification of *Sarcocystis* isolates were performed using PCR-RFLP based on the authors` previous study (11). The *18srRNA* gene sequence was mined from genbank and the specific primer pair was designed using Primer3 software that included sarF 5'- CGT GGT AAT TCT ATG GCT AAT ACA - 3' and sarR 5'-TTT ATG GTT AAG ACT ACG ACG GTA-3'. The amplicon size was estimated around 900 base pair. Amplification was performed using 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM each dNTP, 1U of Taq DNA polymerase, 10 pmol of each primer and 100ng of the genomic DNA as a template. This amplification was performed for the negative control including all the reagents but not the template. The amplification program was done with an initial denaturation of 94°C for five minutes, followed by 30 cycles of 94°C for 60 seconds, 58 °C for 60 seconds and 72°C for 60 seconds and it was finalized with the extension of 72°C for five minutes. The amplicon was analyzed on 1% agarose gel using electrophoresis, alongside with 100 bp of the DNA ladder.RFLP analysis was performed using *BfaI* and *RsaI* restriction enzymes (11). The reaction was carried out with 10U either *BfaI* or *RsaI* restriction enzyme, 1X specific buffer and 10μL of the PCR products, then, was incubated for 16 hours at 37°C according to the manufacturer's recommendations. The digestion was analyzed, using agarose gel electrophoresis alongside the 100 bp DNA ladder.

4. Results

Among 25 commercial hamburger samples, 17 (%68) showed a PCR product around 900 bp which could detect *Sarcocyst Spp*. RFLP analysis was performed using *BfaI* and *RsaI* restriction enzymes and it was analyzed using the agarose gel electrophoresis. 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 bp detected *S. cruzi*. The restriction enzyme of *RsaI* was used in order to distinguish between *S. hominis* and *S. hirsuta*. After RFLP with *RsaI*, the restriction fragments of 376 bp and 557 bp detected *S. hirsuta* and fragment of 926 bp, without any digestion, detected *S. hominis* (Figure 1). For verification, each species detected in the samples were randomly selected and sent for sequencing and the results were analyzed with BLAST.

Figure 1. PCR-RFLP to Identify *S. hominis*



Lane 1, 100 bp DNA ladder; Lane2, digestion with *RsaI*, no digestion; Lane3, digestion with *BfaI* resulting two fragments of 376bp and 550bp; Lane4, PCR product before any digestion with 926 bp in size.

5. Discussion

The family *sarcocyst* includes cyst-forming coccidia, which parasitize a wide variety of vertebrates including humans. *Sarcocystis* species are among the most common and widespread protozoan parasites of livestock with economic importance around the world. A large number of vertebrate species serve as intermediate or definitive hosts for *Sarcocystis* species, and some hosts are often infected by several different *Sarcocystis* species at the same time. Such mixed infections lead to taxonomic confusion within the genus *Sarcocystis*. This confusion is compounded by incomplete knowledge of the life cycles of many *Sarcocystis* species and the paucity of informative morphological characters for apicomplexans in general. The species are distinguished by the structure of the cyst wall under light and transmission electron microscopies (12).

In all previous surveys, impression smear, histological or digestion techniques were used to detect *Sarcocystis spp*. Since none of the indicated assays could identify *S. hominis* and *S. hirsute* (13, 14), the prevalence of these species in the cattle population of Iran was not reported earlier. Therefore, designing and development of a sensitive and specific protocol could have an important role to detect and identify *Sarcocystis spp*. and report the true prevalence of this zoonotic pathogen (11). The current study used *18S rRNA* as a suitable target because this region has little interspecific variation but high sequence divergence between the species (15). This variable in the region could make it suitable to identify species. Previously, there were some documents for molecular detection and identification (16, 17) but the current design introduced just two restriction enzymes for identification that is cost-effective especially for developing countries (18, 19). As shown in the results, the designed primer could amplify an amplicon around 900 bp and the restriction digestion analysis with *BfaI* showed 376 bp and 397 bp restriction fragments for *S. hominis* or *S. hirsuta* and 184bp, 371 bp and 382 bp for *S. cruzi*. The restriction enzyme of *RsaI* was used in order to distinguish between *S. hominis* and *S. hirsuta*. After RFLP with *RsaI*, 376 bp and 557 bp restriction fragments detected *S. hirsuta* and 926 bp fragment, without any digestion, detected *S. hominis*. In conclusion, the current study developed a practical technique which could detect prevalence of *S. hominis* in meat products such as hamburgers.

Acknowledgements

The authors wish to thank the authorities of Shahid Sadoughi University of Medical Sciences for their financial support. Also, we thank from Mr. Ali Amini and Mrs. Farzaneh Mirzaei for their help.

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