Molecular Method Development to Identify Foodborne \textit{Sarcocystis hominis} in Raw Beef Commercial Hamburger

Bahador Hajimohammadi \textsuperscript{1,2}; Mahsa Moghadam Ahmadi \textsuperscript{2}; Gilda Eslami \textsuperscript{2,3,4,\ast}; Ahmad Oryan \textsuperscript{4}; Ali Dehghani \textsuperscript{3,\ast}; Amin Zohourtabar \textsuperscript{2}

\textsuperscript{1}Research Centre for Molecular Identification of Food Hazards, Shahid Sadoughi University of Medical Sciences, Yazd, IR Iran
\textsuperscript{2}Department of Food Hygiene and Safety, Faculty of Health, Shahid Sadoughi University of Medical Sciences, Yazd, IR Iran
\textsuperscript{3}Department of Parasitology and Mycology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, IR Iran
\textsuperscript{4}Department of Pathology, School of Veterinary Medicine, Shiraz University, Shiraz, IR Iran
\textsuperscript{\ast}Department of Biostatistics and Epidemiology, Faculty of Health, Shahid Sadoughi University of Medical Sciences, Yazd, IR Iran

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Background: \textit{Sarcocystis spp.} is a zoonotic parasitic pathogen endangering safety of meat and derived meat products such as hamburgers which are among the most popular fast foods worldwide.

Objectives: The current study aimed to design a protocol for molecular identification of \textit{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\degree C for the next steps. The genomic DNA was extracted using salting out method. Detection and identification of \textit{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 polymorphisms (RFLP) analysis was performed using \textit{BfaI} and \textit{RsaI} restriction enzymes. The digestion was analyzed, using agarose gel electrophoresis alongside 100 base pair DNA ladder.

Results: Among 25 commercial hamburger samples, 17 samples showed a PCR product around 900 bp which could detect \textit{Sarcocystis Spp.} After RFLP with \textit{BfaI}, the restriction fragments of 376 bp and 557 bp detected \textit{S. hominis} or \textit{S. hirsuta} and fragments of 184 bp, 371 bp and 382 bp detected \textit{S. suihominis}. After RFLP with \textit{RsaI}, the restriction fragments of 376 bp and 537 bp detected \textit{S. hirsuta} and fragment of 926 bp, without any digestion, detected \textit{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 \textit{S. hominis} in meat products such as hamburgers.

Keywords: \textit{Sarcocystis spp.; Food borne Diseases; Molecular Diagnostic Techniques}

1. Background

\textit{Sarcocystis spp.} as the obligatory intracellular two-host parasites belong to the family of \textit{Sarcocystidae} and phylum of \textit{Apicomplexa} (1, 2). Among the numerous species, just \textit{S. hominis} and \textit{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). The pathogenic 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 \textit{Sarcocystis} species in some methods with similar morphology, therefore, the techniques based on DNA analysis will be useful to identify any organisms (9), also

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 \textit{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. Gross examination, impression smears, and digestion methods are the usual methods in his to pathological methods. Gross examination, impression smears, and digestion methods are the usual methods in his to pathological methods. Gross examination, impression smears, and digestion methods are the usual methods in his to pathological methods. Gross examination, impression smears, and digestion methods are the usual methods in his to pathological methods. Gross examination, impression smears, and digestion methods are the usual methods in his to pathological methods. Gross examination, impression smears, and digestion methods are the usual methods in his to pathological methods. Gross examination, impression smears, and digestion methods are the usual methods.

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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 18S rRNA.

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 30 mg of each sample was suspended in 900 μL 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 100 μL 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 100 ng 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 Sarcocystis 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 557bp; Lane4, PCR product before any digestion with 926 bp in size.
5. Discussion

The family sarcocyst includes cyst-forming coccidia, which parasite a wide variety of vertebrates including humans. Sarcocystis species are among the most common and widespread protozoa 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. hirsuta (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 interspecies 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 and S. hirsuta and 184bp, 371 bp and 382 bp for S. cruzi. The restriction enzyme of Rsal was used in order to distinguish between S. hominis and S. hirsuta. After RFLP with Rsal, 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.

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