

Prevalence and species identification of *Sarcocystis* in raw hamburgers distributed in Yazd, Iran using PCR-RFLP

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ABSTRACT

Introduction: Hamburger is a popular type of fast foods consumed all over the world. *Sarcocystis* spp. is a zoonotic parasitic pathogen which endangers safety of meat and meat products. The present study describes the prevalence rate of *Sarcocystis* spp. in hamburgers in Yazd, Iran using PCR-RFLP.

Materials and methods: Raw hamburger samples (100 traditional and 90 industrial) from central region of Iran, Yazd were randomly selected. The genomic DNA was extracted using salting out method. Detection and identification of *Sarcocystis* isolates was performed using PCR-RFLP.

Results: The results showed that 77.9% of all tested hamburger samples were infected with *Sarcocystis* spp. The infection rate in the traditional hamburger (87%) was significantly ($p < 0.05$) higher than the industrial ones (67.8%). The rate of *S. cruzi*, *S. hirsuta* and *S. hominis* in the traditional hamburger samples, was 39%, 61% and 54% respectively; while the rate of *S. cruzi*, *S. hirsuta* and *S. hominis* in the industrial hamburgers was 67.8%, 58.9% and 57.8%. *S. cruzi*, respectively. The rate was significantly ($p < 0.05$) higher in industrial hamburgers than in the traditional ones. No statistical association was found between percentage of meat content and the rate of contamination in the industrial hamburger ($p > 0.05$).

Conclusion: Having zoonotic significance, considerable infestation rate of *S. hominis* seems to be high in hamburgers of this region of Iran. To the best of our knowledge, this is the first of its own kind carried out in hamburger samples in Iran.

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Introduction

Hamburger is a popular type of fast foods produced and consumed all over the world. It is estimated that about five billion hamburgers are consumed just in USA annually (Prayson et al., 2008). There are no data on per capita of meat products use in Iran, but, consumption of hamburger is considerable in food diet of Iranian people, especially in those who work outside home because of easy and fast preparation of the food as well as its favorable taste and cost.

Iranian hamburger is mainly comprised of meat, onion, wheat

flour, garlic, edible oils, vegetable protein, pepper, table salt and occasionally traditional spices. It should be highlighted that similar to some other Islamic countries, pork is not used as meat ingredient in production of hamburgers in Iran, and beef is the major meat utilized to prepare this product.

Iranian hamburgers are divided to industrial and traditional types. The Industrial hamburgers are produced in meat processing plants under supervision of Iranian Health and Medical Education Ministry.

According to national standards hamburgers are categorized into three kinds based on the meat content as 30%, 60-74% and 75-95% (ISIRI, 2007). Traditional hamburger is a home-made meat product which is prepared and sold in the street markets without any considerable safety surveillance and not according

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the national standard. So, due to high consumption of these meat products, the hygiene and safety is of crucial importance from the public health viewpoint.

Sarcocystis spp. is a zoonotic parasitic pathogen which endangers safety of meat and meat products. The genus *Sarcocystis* is comprised of about 130 species of intracellular protozoan coccidia that belongs to family Sarcocystidae and phylum Apicomplexa (Nematollahia et al., 2013; Nourollahi-Fard et al., 2009; Oryan et al., 1996; Xiang et al., 2009).

These parasites have an obligate two-host life cycle in which sexual and asexual stages occur in carnivore final hosts and herbivore intermediate hosts, respectively. Oocysts are expelled in the feces of final hosts and can infect intermediate hosts in which sarcocysts are formed after several developmental stages in skeletal and cardiac muscles (Bucca et al., 2011; Ghisleni et al., 2006; Oryan et al., 2010; Oryan et al., 2011). There are three species of *Sarcocystis* which infect cattle as intermediate host; *Sarcocystis cruzi*, *S. hirsuta* and *S. hominis* which canids, felids and humans are the final hosts, respectively (Domenis et al., 2011; Fayer, 2004). It is indicated that in many regions of the world, more than 90% of the cattle population are infected to *Sarcocystis* and this makes it as one of the most prevalent parasitic agent in cattle (Jehle et al., 2009; More et al., 2011; Nourollahi-Fard et al., 2009; Nourollahi-Fard et al., 2013; Obijiaku et al., 2013).

Among the *Sarcocystis* species of cattle, only *S. hominis* is zoonotic and infects humans after consumption of infected raw or undercooked beef or derived meat products. The clinical signs of the intestinal *Sarcocystis* in human are digestive system disturbances such as nausea, vomiting, stomachache and diarrhea (Bucca et al., 2011; Fayer, 2004, Nematollahia et al., 2013).

As beef is the main ingredient of hamburger, the occurrence of *Sarcocystis* spp. in this product should be marked. So far, a few reports in regard of *Sarcocystis* infection in hamburgers (Hosseini et al., 2007; Jahed Khaniki and Kia, 2006; Nematollahia et al., 2013; Prayson et al., 2008; Rahdar and Salehi, 2011). To our knowledge there is no information in the literature regarding molecular identification of *Sarcocystis* species in Iranian hamburger. Hence, the present study describes the prevalence rate of *Sarcocystis* spp. in hamburgers in Yazd, Iran with special emphasis on zoonotic importance of *S. hominis*.

Materials and methods

Sample collection

This study was carried out in Yazd, the capital of Yazd Province located in central part of Iran with dry and hot climate. From April to July 2013, a total of 190 raw hamburger samples, 100 traditional and 90 industrial, were randomly collected from

supermarkets and street food sellers in city of Yazd, Iran. The industrial hamburgers were randomly obtained based on percentage of the meat content indicated in the package labels. In laboratory, all of the samples were cut into 5 mm slices and examined carefully by naked eye for the presence of macrocysts of *Sarcocystis*. Five slices from different parts of each hamburger sample were selected and mixed well, and then preserved in ethanol 70% at -20 °C for the next steps.

DNA extraction

The genomic DNA was extracted using salting out method. Briefly, approximately 30 mg of each sample was suspended in lysis buffer containing 900 µl NET buffer (50mM NaCl, 25mM EDTA pH 8.0, 50mM Tris-HCL pH 7.6), with 10 µl proteinase K (20 mg/ml, Fermentas, EO049), and SDS with end concentration of 1% followed by rapid mixing and incubation at 56 °C for an overnight. The lysate was treated with 250 µl 6M NaCl. After centrifugation, the supernatant was transferred into a new sterile 1.5 ml microtube. The DNA precipitation was performed by adding double volume of chilled absolute ethanol. After washing with ethanol 70%, the DNA pellet was dried and resuspended in 100 µl sterile double distilled water, aliquoted and stored at -20°C.

Verification of the beef in the traditional hamburgers

Traditional hamburgers have no package labeling and the meat ingredient may be other than beef (such as mutton). Therefore, to ensure the presence of beef, we amplified the beef *cytchrome b* gene using specific primers of cytbF 5'-CTG CCT AAT CCT ACA AAT CCT C -3' and cytbR 5'-CGT AAT ATA AGC CTC GTC CTA C-3'. Amplification was performed using 1X PCR buffer, 1.5 mM MgCl₂, 0.2 mM each dNTPs, 1U of Taq DNA polymerase, 10 picomol solution of each primer and 100 ng genomic DNA as a template in a 25µl PCR reaction mix. The PCR assay was performed in VerPlex (ABI) with a heated lid. The cycling conditions included an initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec and finalized with extension of 72 °C for 5 min. This amplification was performed for negative control contained all reagents but no template. Presence of an amplicon with 200bp verifies the beef meat. Analysis of the amplification results was done using agarose gel electrophoresis alongside with 100 bp DNA ladder. Finally, the presence of beef in all of the traditional hamburgers was verified and the samples were kept for species detection of *Sarcocystis* isolates using molecular technique of PCR-RFLP.

Detection and identification

Detection and identification of *Sarcocystis* isolates was perfor-

med using PCR-RFLP. The amplification of the target gene, *18s rRNA* gene was done using specific primers of sarF 5'-CGT GGT AAT TCT ATG GCT AAT ACA-3' and sarR 5'-TTT ATG GTT AAG ACT ACG ACG GTA-3' (Yang et al., 2002). Based on databases, the amplicon size of *S.hominis*, *S. hirsuta* and *S. cruzi* is 926 bp, 953 bp and 937 bp, respectively. Amplification was performed, using 1X PCR buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1U of Taq DNA polymerase, 10 pmol each of primers and 100 ng genomic DNA as a template. This amplification was performed for negative control contained all the reagents but no template. The amplification program was done with an initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C for 60 sec, 58 °C for 60 sec and 72 °C for 60 sec and finalized with extension of 72 °C for 5 min. The amplicon was analyzed on 1% agarose gel using electrophoresis, alongside with 100 bp DNA ladder.

RFLP analysis was performed using *Bfal* and *Rsal* restriction enzymes. The reaction was carried out with 10 U either *Bfal* or *Rsal* restriction enzyme, 1 X specific buffer and 10 µl PCR products.

Then, it was incubated for 16 h at 37 °C according to the manufacturer's recommendations. The digestion was analyzed, using agarose gel electrophoresis alongside of 100 bp DNA ladder.

After RFLP with *Bfal*, the restriction fragments of 376 bp and 397 bp detected *S. hominis* or *S. hirsuta* and fragments of 184bp, 371bp and 382 bp detected *S. cruzi*. The restriction enzyme of *Rsal* was used in order to distinguish between *S. hominis* and *S. hirsuta*. After RFLP with *Rsal*, the restriction fragments of 376bp and 557bp detected *S. hirsuta* and fragment of 926bp, without any digestion, detected *S. hominis*. For verification, each species detected in the samples was randomly selected and sent

for sequencing and the results were analyzed with BLAST. The isolate regarding to *S. cruzi* is getting indexed in genbank with accession number of KF933850.

Statistical analysis

The statistical analysis was performed by Chi-Square, using SPSS software (v. 16.0.), and the $p < 0.05$ level was considered significant.

Results

Among 190 hamburger samples, 77.9% were infected by *Sarcocystis* spp.; however no macroscopic sarcocysts was found. The infection rate in the traditional hamburger (87%) was significantly ($p < 0.05$) higher than the industrial ones (67.8%).

The rate of *S. cruzi*, *S. hirsuta* and *S. hominis* in the traditional hamburger samples, was 39%, 61% and 54%, respectively; while the rate was 67.8%, 58.9% and 57.8% in the industrial hamburgers (Table 1).

S. cruzi was significantly more prevalent in industrial hamburgers in comparison with the traditional ones ($p < 0.05$). Occurrence of more than one species in the industrial and traditional hamburgers was respectively 58.9% and 55% with no significant difference ($p > 0.05$).

The prevalence rate of *Sarcocystis* species showed no statistical correlation with geographical regions of the sampling from north, south, west, east and central parts of Yazd ($p > 0.05$). Also, no statistical association was found ($p > 0.05$) between the content of meat percentage and the infection rate of industrial hamburgers (Table 2). PCR-RFLP analysis of the species identification of *Sarcocystis* is shown in Fig. 1 and 2.

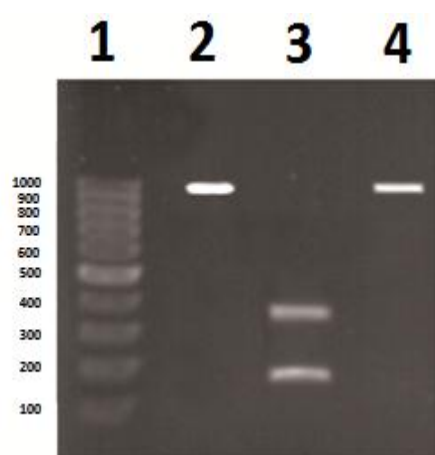


Fig. 1: PCR-RFLP analysis. Lane 1: 100 bp DNA ladder; Lane 2: PCR product of target gene; Lane3: RFLP with *Bfal* (184 bp, 371 bp and 382 bp for *S. cruzi*); Lane 4: RFLP with *Rsal* (no digestion for *S. cruzi*)

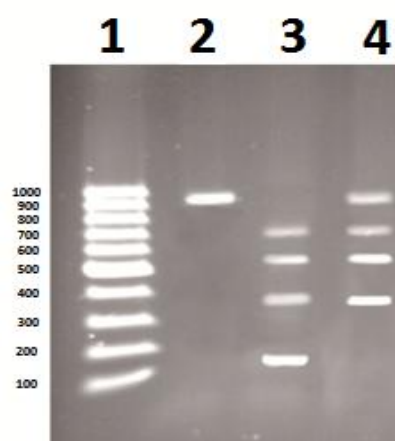


Fig. 2: PCR-RFLP analysis. Lane 1: 100 bp DNA ladder; Lane 2: PCR product of target gene; Lane 3: RFLP with *Bfal* (184 bp, 371 bp and 382 bp for *S. cruzi*, 376 bp and 550 bp for *S. hominis*, 397 bp and 557 bp for *S. hirsuta*); Lane 4: RFLP with *Rsal* (376 bp and 577 bp for *S. hirsuta*, and no digestion for *S. cruzi* and *S. hominis*)

Table 1: *Sarcocystis* spp. infection in the traditional and industrial hamburgers in Yazd, Iran

Sample size	Traditional	Industrial	Total
	100	90	190
<i>S. cruzi</i>	26 (26)	8 (8.9)	34 (17.9)
<i>S. hirsuta</i>	6 (6)	0 (0)	6 (3.2)
<i>S. hominis</i>	0 (0)	0 (0)	0 (0)
<i>S. cruzi</i> and <i>S. hirsuta</i>	1 (1)	1 (1.1)	2 (1)
<i>S. cruzi</i> and <i>S. hominis</i>	0 (0)	0 (0)	0 (0)
<i>S. hirsuta</i> and <i>S. hominis</i>	42 (42)	0 (0)	42 (22.1)
<i>S. cruzi</i> and <i>S. hirsuta</i> and <i>S. hominis</i>	12 (12)	52 (57.8)	64 (33.7)
Total	87 (87)	61 (67.8)	148 (77.9)

Table 2: *Sarcocystis* spp. infection in the industrial hamburgers of Yazd, Iran according to percentage of meat content (30, 60-74 and 75-95%)

Sample size	30%	60-74%	75-95%	Total
	32	30	28	90
<i>S. cruzi</i>	2 (6.3)	2 (6.7)	4 (14.3)	8 (8.9)
<i>S. hirsuta</i>	0 (0)	0 (0)	0 (0)	0 (0)
<i>S. hominis</i>	0 (0)	0 (0)	0 (0)	0 (0)
<i>S. cruzi</i> and <i>S. hirsuta</i>	1 (3.1)	0 (0)	0 (0)	1 (1.1)
<i>S. cruzi</i> and <i>S. hominis</i>	0 (0)	0 (0)	0 (0)	0 (0)
<i>S. hirsuta</i> and <i>S. hominis</i>	0 (0)	0 (0)	0 (0)	0 (0)
<i>S. cruzi</i> and <i>S. hirsuta</i> and <i>S. hominis</i>	18 (56.3)	16 (53.3)	18 (64.3)	52 (57.8)
Total	21 (65.7)	18 (60)	22 (78.6)	61 (67.8)

Discussion

Detection and identification of pathogens in food comprising vegetable, meat and etc. is crucial for public health (Jamali and Banihashemi, 2013). There are various methods to detect and identify the pathogens in food products but molecular method is more precise (Xiao et al., 2009).

There are publications on prevalence of *Sarcocystis* spp. in cattle slaughtered in various areas of the world, indicating high occurrence of *S. cruzi* (Bucca et al., 2011; Ghisleni et al., 2006; Nourani et al., 2010; Nourollahi-Fard et al., 2009; Nourollahi-Fard et al., 2013). However, the applied methods in most of the previous studies are mainly traditional techniques including impression smear, histology and digestion methods, whereas these methods could only identify *S. cruzi*, not *S. hominis* and

S. hirsuta. There are a few reports on the prevalence rate of *S. hominis* and *S. hirsuta* in cattle using electron microscopy or molecular assays (Domenis et al., 2011; Dubey et al., 1989; Jehle et al., 2009; More et al., 2011). In addition, according to the scientific literature database, from those investigations which have been undertaken to detect *Sarcocystis* spp. in hamburgers, none of them has successfully detected different species of *Sarcocystis* because the applied methods were not efficient to identify the species of this parasite (Hosseini et al., 2007; Jahed-Khaniki and Kia, 2006; Nematollahia et al., 2013; Prayson et al., 2008; Rahdar and Salehi, 2011). In this study, PCR-RFLP was applied to identify different species of *Sarcocystis* in the hamburger samples.

Prayson et al. (2008) found *Sarcocystis* spp. in two out of eight examined hamburger brands in USA by using histological

method. Hosseini et al., (2007) reported occurrence of 47.9% (56 of 117) *Sarcocystis* infection, using impression smear assay, in hamburger samples distributed in Tehran, Iran; but the authors did not identify the *Sarcocystis* species in the samples. Another similar study, using digestion method, indicated an infection rate of 56.0% *Sarcocystis* infection in hamburgers in Ahvaz, southern Iran (Rahdar and Salehi, 2011). Recently, Nematollahi et al. (2013) using both impression smear and peptic digestion methods, announced that the prevalence rate of *Sarcocystis* spp. in both traditional and industrial hamburger of Tabriz, northwest of Iran, was the same as 56.25%. It should be highlighted that the infection rate is not only attributed to the geographical area or age and gender of the intermediate host but it is also crucially influenced by the method applied to detect *Sarcocystis* infection. While both the impression smear and peptic digestion methods showed infection rates of 47.9 to 56.0% at various parts of Iran, Jahed-Khaniki and Kia (2006) reported an infection rate of 6.25%, using histological method, in Garmsar, Iran.

The infection rate of 77.9% reported in the present study is much higher than those of the previous studies, because the PCR-RFLP, used in the present study, is a gold method and able to detect small traces of DNA of *Sarcocystis* species in a specimen. PCR-RFLP is the most sensitive assay to detect the *Sarcocystis* spp. compared with the other methods (Jehle et al., 2009; More et al., 2011).

Hosseini et al. (2007) and Nematollahia et al. (2013) found no statistical difference between the infection rate of the industrial and traditional hamburgers. However, in the present study, the prevalence rate of *Sarcocystis* spp. in the traditional hamburgers (87%) was significantly higher than the industrial ones (67.8%) ($p < 0.05$). This difference can be attributed to the fact that in preparing the traditional hamburgers in Yazd which normally is a mixture of beef with various animal origin. But, in the case of the industrial hamburger produced in meat processing plants, whole carcass pertaining to one cattle in most instances is used for preparation of a batch of hamburgers.

Lack of a significant difference in the infection rate of *Sarcocystis* in hamburgers distributed at various regions of Yazd city, is expected; because there is only one slaughterhouse in the city and all the hamburger producers supply their daily required beef from the same slaughterhouse.

Absence of the macroscopic sarcocysts in the present study is in agreement with most of the previous studies (Jahed-Khaniki and Kia, 2006; Nematollahia et al., 2013; Rahdar and Salehi, 2011). It seems the main reason for lack of macrocyst of *Sarcocystis* is related to the observation and condemnation of macrocysts by the meat inspectors during official inspection in the slaughterhouse.

The prevalence rate of *S. cruzi*, in the present study, was significantly higher in the industrial hamburgers compared with

the traditional ones. Since, the authors were not able to find similar molecular study in hamburger; we could not present a comprehensive comparison and analysis on this issue. However, this may be related to combining the imported beef from other countries with meat obtained from slaughtered native cattle. Some meat processing plants combine the native meat with the imported beef in their industrial hamburgers. However, more examinations should be done on the imported beef to explain and clarify this matter.

Conclusion

Based on the present findings, regarding the high prevalence of *S. hominis* in hamburgers, there is a great risk of infection in Iranian population who consume this meat product. Having zoonotic significance, considerable infestation rate of *S. hominis* must be highlighted in Iran. Since there is no published information regarding the incidence rate of intestinal sarcocystosis in Iranian people, the fecal sample of those humans who are at high risk should be examined for sporocysts or oocysts of *S. hominis*. The people who consume semi-cooked hamburger must be trained about the life cycle, public health importance and pathogenicity of this parasite. To achieve an acceptable safety assurance and to inactivate the sarcocysts present in hamburgers, this fast food should be cooked fully to a core temperature of 70°C or be kept at -20 °C for 1 day or -4 °C for 2 days before consumption.

To the best of our knowledge, this study seems to be the first of its own kind carried out in hamburger samples of Iran. More detailed studies are needed to describe the distribution pattern and species identification of *Sarcocystis* spp. in other meat products. Also, in future, detailed studies should be designed to achieve more effective and applied approaches for inactivation of this parasite in hamburgers.

Conflicts of interest

The authors declare no conflicts of interest.

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