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Prevalence and molecular analysis of *Sarcocystis* species infection in slaughtered cattle in Alborz, Iran

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ABSTRACT

Several species of *Sarcocystis* as an obligatory intracellular protozoon have been identified in cattle, including *Sarcocystis cruzi, Sarcocystis hirsuta, and Sarcocystis hominis*, whose definitive hosts are canids, felids, and humans respectively; these zoonotic parasitic species impose a financial and health burden on the livestock industry annually. The aim of the present study, which was conducted for the first time in Alborz province, was to determine the species of *Sarcocystis* based on macroscopic observation, digestion method and restriction fragment length polymorphism (RFLP)-PCR in slaughtered cows in this province in order to complete the information puzzle of the prevalence of Sarcocystis species in Iran and the world cattle.

In the present cross-sectional study, totally 138 samples of slaughtered cows in Alborz province industrial slaughterhouses were collected from diaphragm muscles and examined by macroscopic, digestive and molecular PCR-RFLP methods. This molecular-based method uses variations in homologous DNA sequences (polymorphism) of a population or species or to determine the precise locations of genes in a sequence. Fifty samples were randomly selected for DNA extraction and molecular analysis and sequencing for species identification. Macroscopic examination of the samples showed no cysts, but according to the digestion test results, 100 % of the samples were infected with *Sarcocystis* microcysts. The results of electrophoresis of PCR products showed a band of about 930 bp. The PCR products were digested with restriction enzyme (BFaI) and their digested pattern was evaluated. The results showed that all 50 samples were infected with *Sarcocystis*. After enzymatic digestion of primary PCR products, it was found that (47/50) 94 % and (3/50) 6 % the samples were infected with *S. cruzzi* and *S. hirsuta* species, respectively. No infection was found with *S. hominis*. Cattle slaughtered in Alborz province are highly infected with the *Sarcocystis* parasite, which can affect public health and animal health. The present study suggests that *Sarcocystis* spp. should be diagnosed by relying on high-precision diagnostic methods in order to improve food safety.

1. Introduction

*Sarcocystis*osis is a worldwide parasitic disease caused by *Sarcocystis* spp. belonging to the phyllium apicomplexa. *Sarcocystis* is an obligate heteroxenous protozoan parasite in which sexual proliferation and oocyst formation occur in the final host intestinal mucosa and asexual proliferation occurs in endothelial cells (schizont stage) and striated muscle cells (*Sarcocystis* stage) in intermediate hosts (Dubey & Sykes, 2021). *Sarcocystis* spp. are the most prevalent parasites in domestic animals and causing severe infection in cattle and sheep hosts (Lindsay & Dubey, 2020). Nowadays, >200 species affecting reptiles, birds and (possibly) fishes as well as mammals including human are described within the genus *Sarcocystis* (Dubey & Sykes, 2021); up to now, at least seven named species affect cattle as intermediate host: *Sarcocystis hirsuta, Sarcocystis cruzi* (also as known *S. bovicanis*), *Sarcocystis hominis* (aka *S. bovihominis*), *Sarcocystis bovifelis, Sarcocystis heydorni, Sarcocystis bovini* and *Sarcocystis rommeli* (Dubey & Rosenthal, 2023). Which the *S. hominis* and *S. heydorni*, using humans and non-human primates as

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definitive hosts, *S. cruzi*, using canids as definitive hosts and *S. hirsuta, S. bovifelis, S. bovini* and *S. rommeli*, either confirmed or predicted to use felids as definitive hosts, while *S. bovifelis* DNA has also been detected in the intestine of mustelids (Dubey & Rosenthal, 2023).

It is noteworthy that some *Sarcocystis* species cause worldwide health concern for human societies (Fayer et al., 2015). In *S. hominis*, humans -as a final host- become infected by consuming the undercooked or raw meat of beef that contains tissue cysts with large amounts of bradyzoites; bradyzoites begin their sexual evolution in the intestinal epithelial cells and eventually form the oocysts (Rubiola et al., 2020). The oocyst wall is usually ruptured and the sporocyst containing the sporozoite is excreted in human feces (Rosenthal, 2021). *Sarcocystis* (especially *S. hominis* which is called *S. bovihominis*) causes cystic lesions in the cattle muscles, which is not considered a serious pathogen for cattle, nevertheless, it is important in two ways, first, the affected meats are unusable and annually imposes a high economic burden on the husbandry industry and *Sarcocystis* infected carcasses disposal cost; second, from the zoonotic aspect, it should be considered that human infection with infected meat occurs worldwide and nausea, abdominal/ stomach pain as well as diarrhea are the dominant clinical manifestations which of course can vary depending on the number of eaten cysts (parasite load) and the human host's immune system status (Rosenthal, 2021). As well, depending on the *Sarcocystis* spices and parasite load, different clinical signs such as fever, tachypnea, anorexia, anemia, tachycardia, weight loss, decreased milk production, abortion, neurological disorders, anorexia, and even death can appear/befalls in animals (Decker Franco et al., 2018). Of other species, *S. hirsuta* causes cysts in bovine muscles, and in cats, the final host, undergoes sexual evolution which is mildly pathogenic (Gjerde, 2016). *S. cruzi* species causes microscopic cysts in bovine muscles and the canines are its final host (Xiang et al., 2011). This parasite is the most pathogenic species of *Sarcocystis* in cattle and causes jaundice, myocardial hemorrhage, pneumonia, hair loss, fever, anorexia, anemia (Kalantari et al., 2013).

Despite the mentioned importance, unfortunately, the microscopic diagnosis of *Sarcocystis* spp. faces challenges due to its thin and transparent wall, which leads to many cases of infection being missed, and it is believed that the reported cases (apparent prevalence) are like the tip of the iceberg compared to the actual number of cases (true prevalence) (Omar Swar & Shnawa, 2021). From an economic point of view, animal husbandry is a global profitable, important and influential industry in the economies of countries (Norouzy et al., 2005; Ahsani et al., 2022). Humanity has long faced the challenge of nutrition, and despite further advances in human civilization and technology, adequate food supply and food safety remain a concern for mankind (Jafari Ahmadabadi et al., 2023). Nowadays, safe nutrition is of particular socio-economic importance and is considered one of the important indicators of the level of civilization and progress of societies because in the comprehensive development of a society, the level of mental and physical health of its people is the determining factor in animal breeding (Nejad et al., 2024; Shokri et al., 2023).

Today, almost all the beef consumed in developed and developing countries is supplied from industrial slaughterhouses, therefore, periodic investigations of the prevalence and determination of *Sarcocystis* species in the meat of slaughterhouses seem necessary; unfortunately, accurate statistics of *Sarcocystis* infection and its dominant species are not available in the densely populated Alborz province. It should be noted that biochemical or digestive tests are incapable of accurate diagnosis and species differentiation (Ahsani et al., 2010). Today, PCR is one of the most routine modern laboratory technologies for the detection of infectious agents in a variety of clinical samples with high sensitivity/specificity, fast, and reliable (Mohammadabadi et al., 2011; Mohammadabadi et al., 2004; Shahdadnejad et al., 2016). Scattered studies are available from around the world and Iran, and this study is reported for the first time from Alborz province. Hence, the present study was aimed to determined *Sarcocystis* species in infected meats in Alborz slaughterhouses by digestion method, microscopic observation of bradyzoites and molecular approaches.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Ethics Committee of Alborz University of Medical Sciences (IR.ABZUMS.REC.1397.199).

2.2. Sampling and macroscopic/microscopic examination

In total 138 samples of bovine slaughtered in industrial slaughterhouses including 69 samples from Karaj Rock slaughterhouse and 69 samples from Kordan slaughterhouse were prepared from diaphragm muscles in Alborz province in 2022. All animals were of local origin and were collected and slaughtered from around the city of Karaj (Alborz Province). The studied cows were all male and about 18 months old. In the slaughterhouse, the muscles were examined for the presence of macroscopic cysts and evaluated in methylene blue-staining by counting *Sarcocystiss* per gram of collected samples according to Kirillova et al. (Kirillova et al., 2018). The morphological assessment of *Sarcocystiss* was carried out in freshly squashed preparations by light microscope (Nikon Corp., Tokyo, Japan) at 40X and 100X magnification as described by Prakas et al. (Prakas et al., 2020). Then the samples were examined for the presence of microscopic cysts in the laboratory by digestive and molecular methods.

2.3. Digestive method

First, 50 g of the diaphragm sample was cut into pieces and digested in digestive solution (hydrochloric acid 37 % 7 mL, Nacl 5 gr, pepsin 2.6 gr and distilled water 500 mL) for 8 h according modified Dubey method (Dubey et al., 1988). Then, the sediment of digestive solution containing bradyzoites released from infected samples was stained with Giemsa stain and he slides were investigated under light microscope to bradyzoites detection.

2.4. PCR method

Out of 138 digestion samples that were microscopically infected with *Sarcocytosis*, 50 samples were also randomly selected for DNA extraction and performing PCR-RFLP. Tissue Genomic DNA Extraction Mini Kit (Yekta Tajhiz Azma Company-Iran) was used according manufacture protocol for bradyzoites DNAs extracting. To ensure the quantity and quality of the extracted DNA, the Nanodrop tool (NanoDrop™ 2000 Spectrophotometer- Thermofisher scientific) and agarose gel were used, which have been approved. Molecular PCR and RFLP-PCR methods were applied to differentiate *Sarcocystis* species. Primers were obtained from previously published studies and verified with the BLAST tool (Hajimohammadi et al., 2014; Sarafraz et al., 2020). Forward (sarF 5′-CGT GGT AAT TCT ATG GCT AAT ACA-3′) and reverse (sarR 5′-TTT ATG GTT AAG ACT ACG GTA-3′) primers synthesized by Bioneer (Korea) were used for the amplification of *Sarcocystis* species which targeted 18 s ribosomal RNA gene in PCR technique. Based on databases, the amplicon size of *S. hominis, S. hirsuta* and *S. cruzi* is 926 bp, 953 bp and 937 bp, respectively. In short, the 25 μL volume PCR reaction was amplified for 35 cycles of denaturation at 94 °C for 45 s, annealing at 54 °C for 60 s and extension at 72 °C for 45 s. Final extension was conducted at 72 °C for 5 min Rahdar and Kardooni (2017). Negative and positive controls were included along with the samples in each PCR run; positive control was provided from the Parasitology Department of Tehran University of Medical Sciences. The PCR product was then electrophoresed on 2 % agarose gel to observe the bands formed by the *Sarcocystis* parasite. Next, in RFLP method, PCR product was digested with restriction enzyme BfaI (Thermo Fisher Scientific Company-US). BFaI enzyme was diluted with sterile distilled water at a ratio of 1:10, For this purpose, 10

μL of the PCR products were incubated with the 2 μL diluted enzyme and 2 μL enzyme buffer at 37 °C for 16 h. Eventually, the obtained fragments were electrophoresed in agarose gel for visualization. To confirm the RFLP-PCR findigs, five samples of the PCR product, were sequenced randomly. Four *S. cruzi* and one *S. hirsute* sequences were deposited to GenBank as accession numbers MN829431-4 and PP126631. According to statistical analysis data were analyzed using SPSS 21 statistical software. Differences in the prevalence of the identified *Sarcocystis* species were evaluated using Chi-squared test.

3. Results

In the macroscopic screening (with the naked eye), no cysts/sign indicative of *Sarcocystis* were observed in 138 beef carcass samples examined in the slaughterhouses of Alborz province. On the contrary, in digestion method, *Sarcocystis* bradyzoites were observed in 100 % of the investigated samples with light microscopy (Fig. 1). In the molecular method (PCR), all 50 randomly selected samples (50/50: 100 %) showed bands of about 930 bp for *Sarcocystis* genus (Fig. 2).

Electrophoresis of PCR_RFLP products showed 150, 350, 550 fragments for *S. hirsuta* and 200 and 400 fragments for *S. cruzi* (Figs. 3 and 4). A total of (47/50) 94 % of the samples were infected with *S. cruzi* and (3/50) 6 % of the samples were infected with *S. hirsuta* species. In five sequenced samples, (4/5) 80 % and (1/5) 20 % belonged to *S. cruzi* and *S. hirsuta*, respectively. Regarding the diagnosis of Sarcocytis species, there was a complete agreement between the molecular and sequencing methods.

4. Discussion

Sarcocystis is a ubiquitous obligatory heteroxenous intracellular parasite (Decker Franco et al., 2018). In addition to the importance of *Sarcocystis* species in human societies, it is principal to correct detection/ identity of *Sarcocystis* species in cattle in order to evaluate their economic and public health importance (Rosenthal, 2021). As we know, cattle are host intermediates of several species of *Sarcocytosis*, including *S. cruzi, S. hirsuta*, and *S. homonis. S. cruzi* is the most prevalent (>90 %) species that has been frequently isolated/ reported from adult cattle in worldwide (Dubey & Rosenthal, 2023). A comprehensive study by Shams et al. has estimated the molecular prevalence of *Sarcocystis* species in cattle at 62.7 % (95 % CI 53–71.5 %) according to statistical analysis means, which is very weighty, similar the present study outcomes, which assessed the prevalence to be high (Shams et al., 2022).

Based on digestion technique, all investigated beef carcass samples were reported as positive for *Sarcocystis*, and according to molecular findings, the predominant species was *S. cruzi*, as expected, and the less common species was *S. hirsuta*. While, surprisingly, no evidence of infection was found in the macroscopic checkup. The first striking point can be the low sensitivity rate of macroscopic inspection in slaughterhouses. In line with our findings, Prakas et al., during the exploration for bovine diaphragm muscle samples, evaluated all the cases scrutinized by the digestive method as infected with *Sarcocystis*, as well as the molecular-based evaluation (with 18S RNA and Cox 1 gene), the species distinguished that the dominant species were *S. cruzi*, S. *bovifelis, S. hirsuta* and *S. hominis* respectively (Prakas et al., 2020). Unfortunately, it must be admitted that the current common screening methods are not adequate and reliable; so, many infected cases are probable to be missed (Omar Swar & Shnawa, 2021). On the other hand, the issue becomes doubly important since *S. hominis* and *S. heydorni* are zoonotic species (Dubey & Rosenthal, 2023).

Several morphological characteristics have been evaluated in the microscopic examinations, such that *S. cruzi* has hair-shaped protrusions, although other species (*S. bovifelis, S. bovini, S. hirsuta* and *S. hominis*) have finger-like protrusions, as well, *S. heydorni* characterized by smooth cyst wall (Rudaitytė-Lukošienė et al., 2018; Bittencourt et al., 2016). But the matter is not that easy and it faces challenges, some species can have similar appearance, and during the manipulation with the needle a change in the morphological characteristics of the species (*S. cruzi*) also occurs (Tang et al., 2023; Rubiola et al., 2024); on top, an expert diagnostic technician is needed. There are reports of

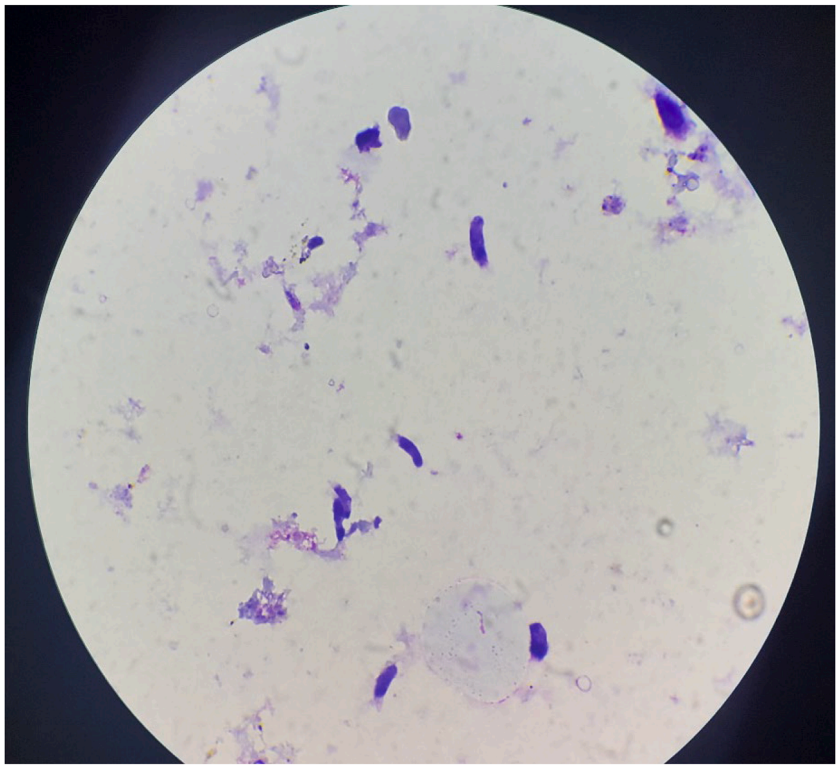

Fig. 1. Sarcocystis parasite bradyzoite observed in Giemsa staining (1000X).

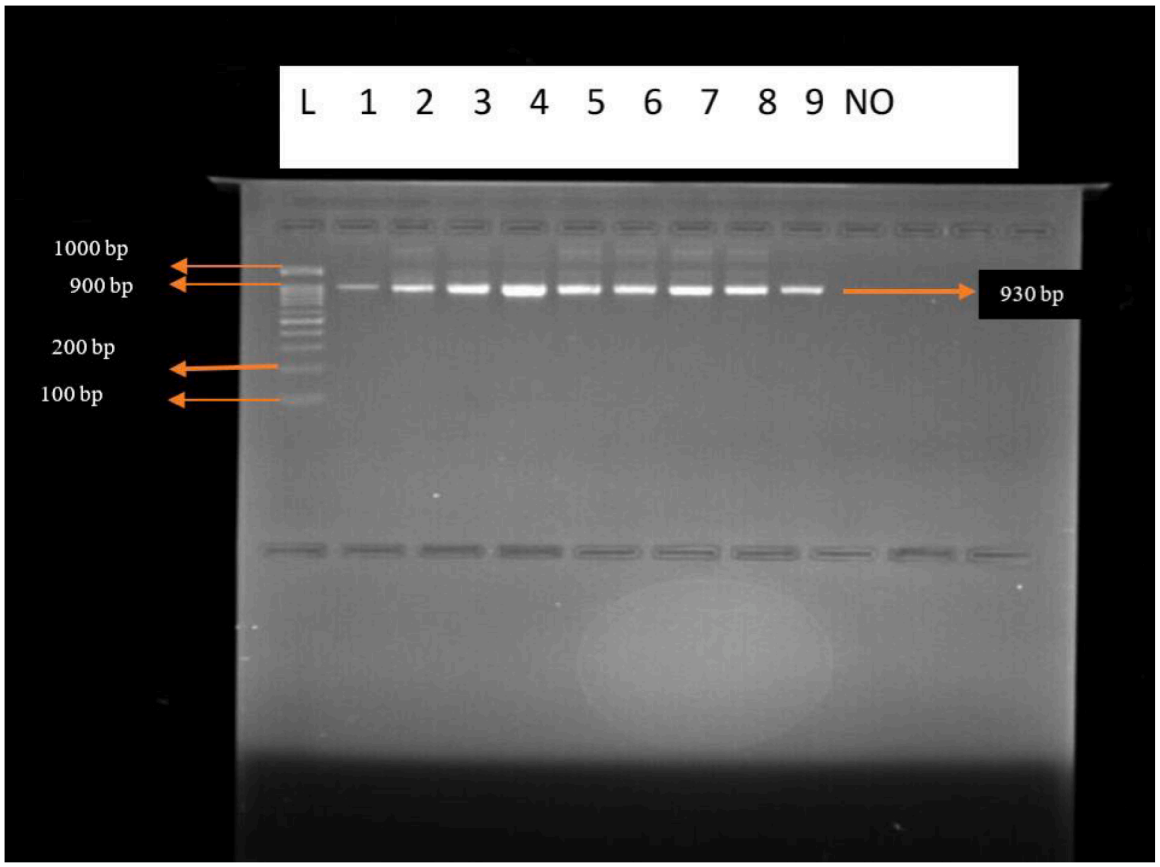

Fig. 2. Electrophoresis of PCR products from 18S rRNA gene amplification on agarose gel (2 %); Column L: Ladder with rate (bp100) Columns 1–8: Bands resulting from *Sarcocystis* amplification 930 bp Column 9 Positive control Column NO: Negative control.

misidentification of *Sarcocystis* species with each other, and according to Prakas et al.'s study, while *S. hominis* was isolated and identified by molecular techniques, it was not seen microscopically; which reinforces these concerns in the diagnosis/ differentiation of *Sarcocystis* species (Prakas et al., 2020). Considering the isolation of *Sarcocystis* from all samples, the digestive method appeared promising that could be beneficial for screening programs and epidemiological investigations (Abdullah, 2021; Dehkordi et al., 2017).

To the best of our knowledge, new molecular approaches have been optimized for more accurate diagnosis, despite their analytical advantages and specificity, it should be noted that some factors affect the sensitivity of these methods, the volume of harvested parasites is vital for DNA extraction because the low parasites load and consequently the obtained DNA can lead to false negative results (Omar Swar & Shnawa, 2021; Sudan et al., 2020). Also, the bias in sampling should be not be neglected, so as to *S. cruzi* is the dominant species in cattle heart samples (Ghaffari et al., 2022). RFLP-PCR is a popular and user-friendly method for the separation of PCR-amplified species, which is useful for *Sarcocystis* (Dameshghi et al., 2023). For the amplification of 18S RNA and Cox 1 genes, according to the results of the present study, 18S RNA gene showed an acceptable discrimination power for all types of *Sarcocystis* (Hoeve-Bakker et al., 2019; Sudan et al., 2021). However, the real-time PCR method seems to be a suitable alternative recently (Moré et al., 2013; Yamazaki et al., 2021). According to the prevalence rate in different geographical regions of Iran, Rahdar and Kardooni's study on abattoir cattle by RFLP-PCR method (with 18srRNA gene) indicated 100 % infection of the samples and in line with our findings, rendering to the sequencing outputs, the dominant species was *S. cruzi* (Rahdar & Kardooni, 2017). In the study by digestion method and RFLP PCR on 290 cows' diaphragm and esophageal specimens in northwestern Iran 87.9 % (253 samples) were infected with *Sarcocystis cruzi* and 1 % (3 samples) were infected with *Sarcocystis homonis* (Sarafraz et al., 2020). As mentioned earlier, the examined sample is effective in the isolated species, in a study conducted in Romania on heart samples of 117 cows, although 17.9 % of the samples were diagnosed as infected with *Sarcocystis* microscopically, using the molecular method, all the isolates were identified as *Sarcocystis cruzi* (Imre et al., 2019). Considering the above interpretations, our findings were consistent with the majority of global reports. Interestingly, documentation of raw meat products infections such as raw hamburgers with *Sarcocystis* spp. is available (Mavi et al., 2020; Hooshyar et al., 2017). Another point that should not be neglected is the transmission of parasites between animals living close to each other, so that the dog's infection can cause transmission to cows both in traditional livestock farming and industrial cattle farms (Yabsley, 2017; Prakas et al., 2021).

The abundance of stray dogs and cats around the villages and grazing areas of cows, as well as the access of these animals to uncooked or raw beef, has established the cycle of transmission of this protozoan between the final and intermediate hosts and causes high infection of cows to Sarcocytosis (Chhabra & Samantaray, 2013; Ford, 1986; Máca et al., 2024). Therefore, cutting off the transmission chain and feeding cats and dogs with cooked and healthy foods can reduce the burden of Sarcocytosis infection (Yabsley, 2017).

Sarcocystis infection in cattle has been reported from most parts of the world, with 87 %, 99.7 %, 100 % and 97.8 % reported in Australia, Germany, the New Zealand and Iraq respectively (Abdullah, 2021; Savini et al., 1992; Moré et al., 2014; Böttner et al., 1987). As mentioned, the findings of the present study are similar to other studies conducted in Iran and the world by digestion. In all studies, high prevalence of *Sarcocystis* in cattle was reported and, in most records, the infection rate was 100 % similar to the present study.

The present study was faced with limitations, the main of which was

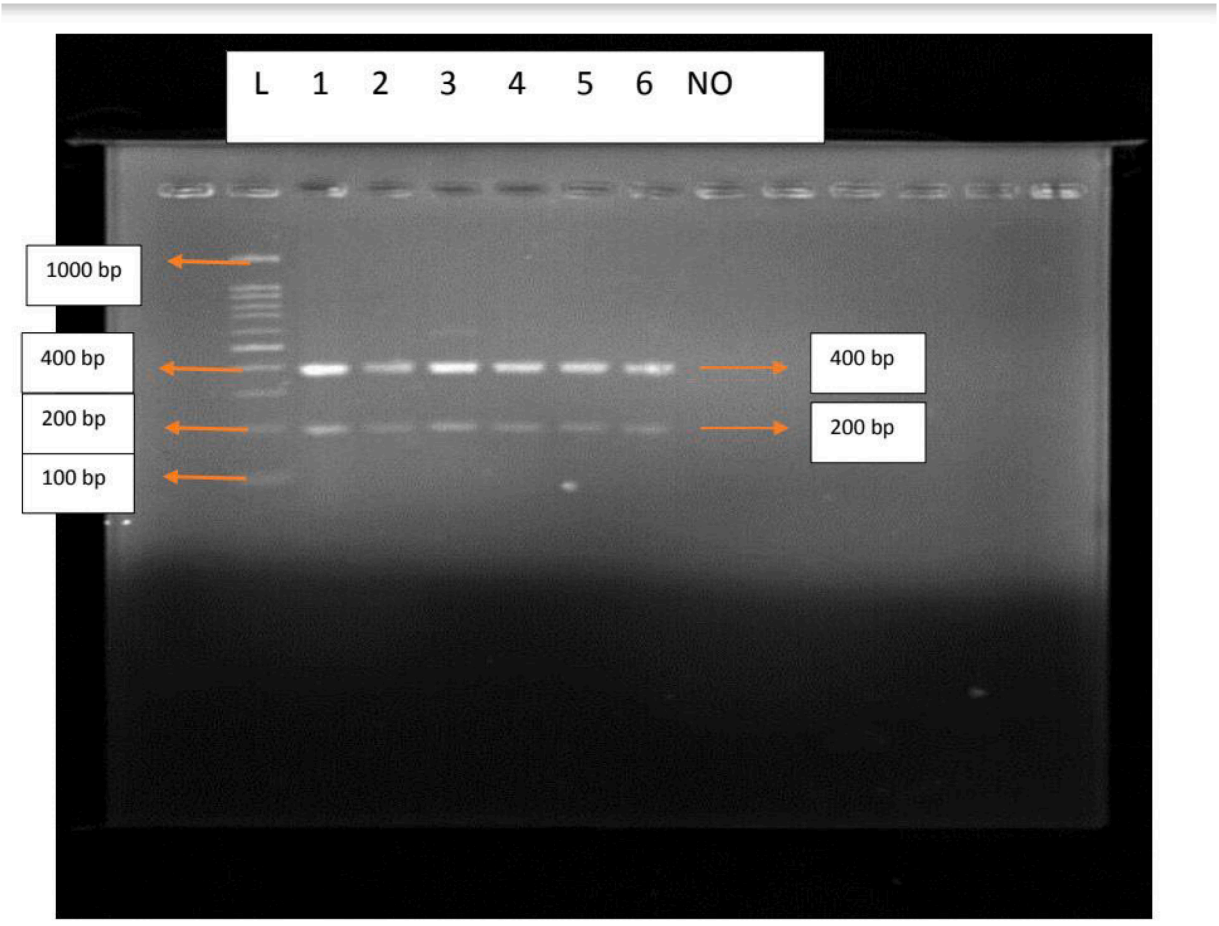

Fig. 3. Enzymatic section of *Sarcocystis* cruciate PCR products using BFaI enzyme on 2 % agarose gel Column L: Ladder with a rate of (100 bp) Column 1–6: Digested samples of Crucifix *Sarcocystis*) Cutting result: a band of about 200 bp and a band of about 400 bp (Column NO: Negative control).

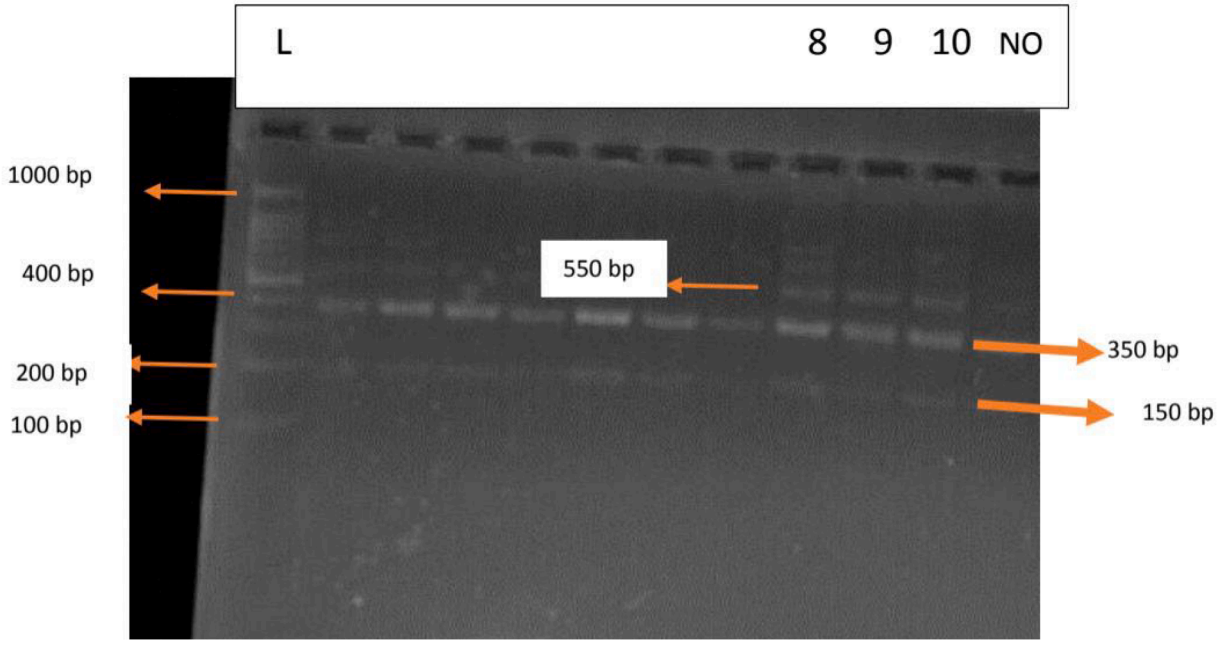

Fig. 4. Enzymatic section of *Sarcocystis hirsuta* PCR products using BFaI enzyme on 2 % agarose gel Column L: Ladder (100 bp) Column 8–10: Digested specimens of *Sarcocystis hirsuta* (cutting result: a band of about 150 bp, a band of about 350 bp, and a band of about 550 bp) Column NO: Negative control.

the impossibility of sequencing all the samples due to the limitation of financial resources, according to the report of few cases of contamination of beef with *S. humonis* in some Iranian studies, it seems that it would have been better if all the samples were sequenced.

5. Conclusion

The present study showed that cows in Alborz province are severely infected with *Sarcocystis* and since macroscopic cysts were not seen in any of the samples, it indicates that conventional methods of visual inspection of carcasses in the slaughterhouse are not able to detect cases of infection. There is a need for digestive parasitology and molecular analysis of meats for *Sarcocystis* hominis, which is capable of causing human infection cases.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Alborz University of Medical Sciences Research Ethics Committee (ethic committee No: IR.ABZUMS.REC.1397.199); there are no participants in this study and consent form is not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

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CRediT authorship contribution statement

Nahid Abdolahi: Writing – original draft, Methodology, Investigation, Data curation. **Aliehsan Heidari:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Amir Bairami:** Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization. **Abolfazl Miahipour:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis. **Monireh Sezavar:** Writing – original draft, Software, Resources, Methodology, Investigation. **Aref Teimuri:** Writing – original draft, Validation, Software, Methodology. **Saeed Bahadory:** Writing – original draft, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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