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Molecular identification of macroscopic cysts of *Sarcocystis* in sheep in Babol, in the north of Iran

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Abstract. *Sarcocystis* is one of the most prevalent parasite in domestic animals in the world. In this study, we examined 50 macroscopic cysts in sheep muscles from Babol, in the north of Iran. Genomic DNA extraction and polymerase chain reaction (PCR) were performed to amplify a 609bp length based on 18S rRNA gene. The results of restriction of *Ava*I, *Hind* II, *Taq*I and *Eco*RI enzymes demonstrated that all the samples were *Sarcocystis gigantea*. The results of this study supports the importance of molecular techniques for characterization of *Sarcocystis* species when valid preventive programs for identification and source of infection and progression of immunological diagnosis strategies are needed.

INTRODUCTION

Sarcocystis spp. are obligatory heterogeneous, with herbivorous as the intermediate hosts and also includes various species of reptiles, birds, small rodents, hoofed animals and carnivorous definitive hosts (Dubey *et al.*, 1989; Dubey 1976). In the past, the taxonomy of *Sarcocystis* species has been based on differences in morphology and life cycle. However, morphological features are often subjected to change during the developmental cycle of the parasites, and the complete life cycle of several organisms currently classified as *Sarcocystis* species are still unknown (Levine 1988). Most infections in intermediate hosts like cattle, sheep, goat, pigs, etc. are less severe and asymptomatic, and are observed during post-mortem examination of carcasses. However, in rare cases of massive infections the animals may develop acute syndrome with nervous signs and abortions (Andrews *et al.*, 1990). It also results in severe economic losses due to clinical and subclinical disease, loss of aesthetic value of meat, condemnation and downgrading of carcass (Adriana *et al.*, 2008). Sheep are intermediate hosts of four *Sarcocystis* species: *Sarcocystis tenella* (synonym *Sarcocystis ovicanis*) and *Sarcocystis gigantea* (synonym *Sarcocystis ovifelis*) are distributed world-wide. *Sarcocystis arieticanis* has been found in Europe, the USA, Australia and New Zealand, while infections with *Sarcocystis medusiformis* have been reported only from Australia and New Zealand (Dubey *et al.*, 1989; Tenter 1995). *S. gigantea* and *S. medusiformis*, which are transmitted by felids, usually develop macroscopically visible cysts and are non-pathogenic. *S. tenella* and *S. arieticanis* are transmitted by canids, develop microscopically visible cysts and are pathogenic (Buxton 1998; Dubey *et al.*, 1989b; Dubey and Lindsay 2006). A primary infection of sheep with one of the pathogenic species may lead to acute disease or abortion during the early phase of infection

and chronic disease during the late phase of infection.

In recent years the introduction of new molecular biological techniques has offered new diagnostic methods for parasitic infections. These include direct diagnostic tests established for the detection of *Sarcocystis* molecules by specific DNA probes, which are a valuable option to indirect tests based on recognition of *Sarcocystis*-specific antibodies, and may compensate the diagnostic limitations caused by delayed seroconversion at the onset of acute sarcocystosis.

The aims of our study were to determine the prevalence of *Sarcocystis* infection in sheep destined for human consumption in Babol, in the north of Iran as well as to accurately identify *Sarcocystis* species that infect sheep using molecular techniques.

MATERIALS AND METHODS

Samples collection and digestion

Through daily visits from 1 July to 1 August 2011 to slaughterhouse in Babol County, sheep muscles from esophagus, diaphragm, intra-costal, and abdomen were examined and 50 macroscopic cysts were collected then transferred to laboratory for performing other diagnostic analyses. The cyst samples were kept at -20°C until examination. All of the macroscopic cysts were separated from residual tissue with scalpel. Then, the cysts containing bradyzoites were washed 3 times with Phosphate-buffered saline (PBS) and preserved at -20°C until DNA extraction.

DNA extraction and PCR amplification

For DNA extraction, a small piece of each sample was selected and DNAs were extracted using tissue DNA extraction kit (Cinnagen Co., Tehran, Iran). Amplification of the 18S rRNA gene was carried out in 50 µl reaction volumes containing 1 µl of DNA template, 5 pmol of reverse and forward primers, 2.0 mM MgCl2, 5.0 µl 10x PCR Buffer, 200 µM of each dNTP and 2.5 U Taq DNA polymerase. Forward and reverse genus specific primer sequences used in this study were (Sar-F1): 5'GCA CTT GAT GAA TTC TGG CA 3' and (Sar-F2): 5'CAC CAC CCA TAG AAT CAA G 3' respectively (Hamidinejat *et al.*, 2014). The thermal program of PCR was as follows: 94°C for 5 min, 30 cycles of 94°C for 2 min, annealing at 57°C for 30 s, and 72°C for 2 min, followed by a final extension step at 72°C for 5 min. The PCR product was loaded on 1% agarose gel, stained with safe stain and visualized on a UV transilluminator. The expected PCR product had a length of 609 bp. For observation of specifity of primers and any possibility of the cross reaction with related protozoans, *Neospora caninum* and *Toxoplasma gondii*, the whole tachyzoites of these two parasites were also amplified. The PCR products were analyzed with RFLP using *AvaI*, *HindII*, *TaqI* and *EcoRI* restriction enzymes. Digested PCR products were resolved with vertical electrophoresis method on a 1.5% polyacrylamide gel (PAGE) and visualized with safe stain under ultraviolet light.

RESULTS

Amplification of *T. gondii* and *N. caninum* tachyzoites by PCR with the mentioned primers showed no electrophoretic bands (Fig. 1) but in the same situation, our isolates demonstrated a *Sarcocystis* specific 609 bp band (Fig. 2). Gel electrophoresis of the PCR–RFLP by amplification of 18S rRNA gene from all isolates of macrocyst of *sarcocysits* showed that restriction with *TaqI* enzyme produced 270 and 339 bp fragments and *HindII* produced 198 and 411 bp fragments (Figs. 3). These results represent the *S. gigantea*.

DISCUSSION

Sarcocystosis is a zoonotic and parasitic disease commonly seen in domestic animals such as buffaloes, cattle, sheep and pigs. It has been reported that, depending on the species and their hosts in many herbivores have the ability to cause considerable pathological damages. Among these, *Sarcocystis suihominis*, *S. hominis* is important in terms of public health.

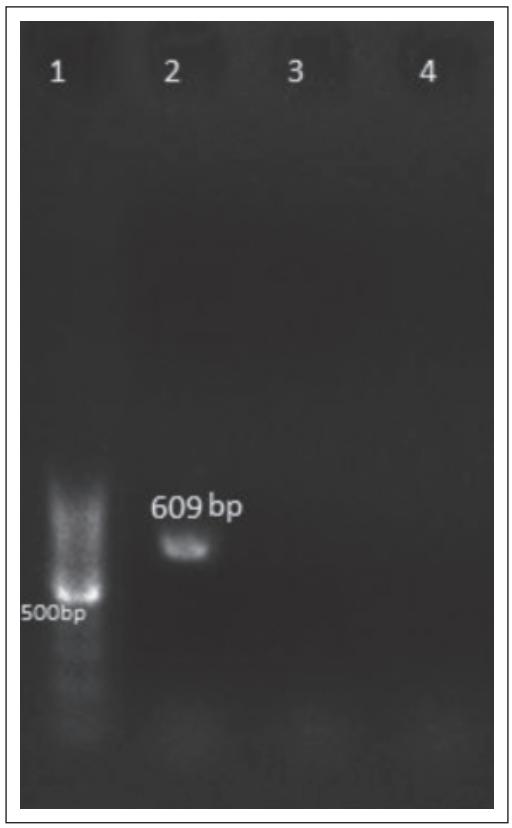

Figure 1. PCR analysis of 18S rRNA gene with *T. gondii*, *N. caninum* and *Sarcocystis* sp. (1) 100 bp ladder, (2) Sheep *Sarcocystis* bradyzoit (3) *T. gondii* tachyzoite, (4) *N. caninum* tachyzoite.

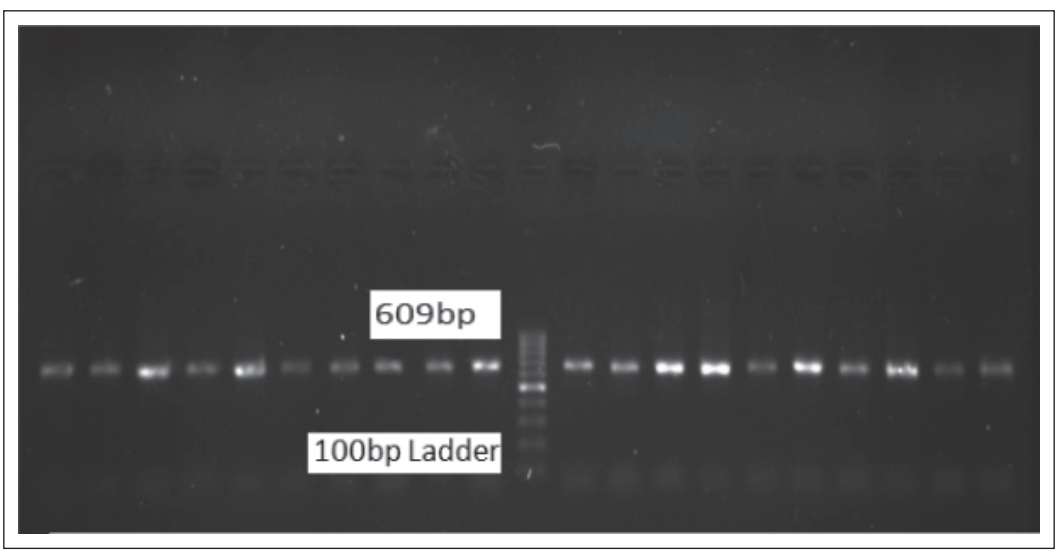

Figure 2. PCR analysis of different isolates of sheep macrocyst of *Sarcocystis*.

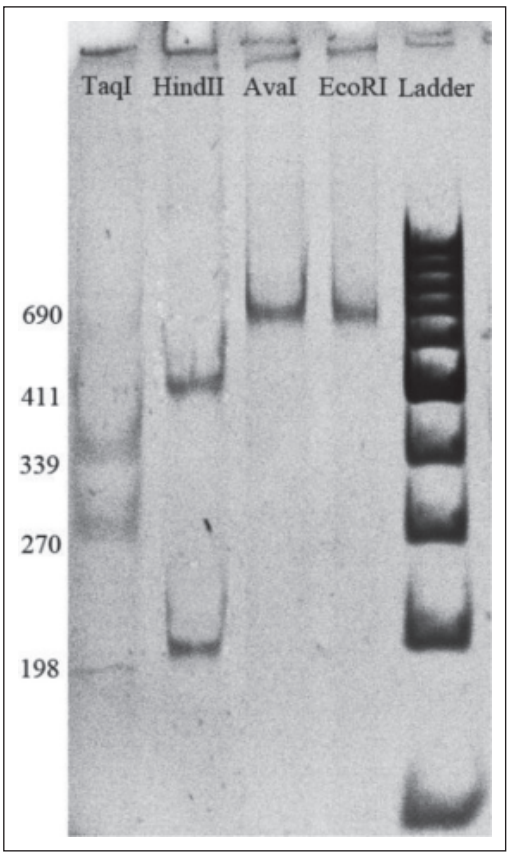

Figure 3. Vertical electrophoresis on polyacrylamide gel. PCR products of the 18S rRNA gene from isolated macrocyst of *Sarcocystis* sp. from examined sheep digested by *EcoRI*, *TaqI*, *HindII* and *AvaI* restriction enzymes.

Previous studies in Iran and the world have indicated that the above mentioned animals can be infected with *Sarcocystis* (Silva RC *et al.*, 2009; Oryan *et al.*, 2010; Fazly *et al.*, 2016; Whaeeb *et al.*, 2016). The high prevalence of *Sarcocystis* infection in slaughtered cattle and sheep is also observed in various studies in different countries that, range of infection between 70 to 100% have been reported (Britt *et al.*, 1990; Woldemeskel *et al.*, 1994 Pena *et al.*, 2001; More *et al.*, 2011; Latif *et al.*, 2015). In Iran, nearly 100% of animals are infected with *Sarcocystis* (Hamidinejat *et al.*, 2014; Dalimi *et al.*, 2008; Borji *et al.*, 2012). Different species of *Sarcocystis* have been isolated from animals in Iran. Dalimi and coworkers found *S. gigantea* and *S. articanis* in sheep using the PCR-RFLP method in Qazvin province (Dalimi *et al.*, 2008). Kia *et al.* reported *S. miescheriana* from boar (Kia *et al.*, 2011). In East Azerbaijan Province, *S. ovicanis* has been reported from sheep (Shahbazi *et al.*, 2013). From what is being observed in the west and southwest (Hamidinejat *et al.*, 2014), as well as Qaem shahr-the north of Iran (not published), it seems that *Sarcocystis gigantea* is the most prevalent species in the majority of areas of Iran.

In the present study, all 50 of the samples collected from sheep muscles were infected with *S. gigantea*. In the food and meat industry, sarcocystosis caused by *S. gigantea* can lead to condemnation of meat and economic losses for farmers (Dubey *et al.*, 1989).

Four *Sarcocystis* species that have been reported in sheep are *Sarcocystis gigantea*, *Sarcocystis arieticanis*, *Sarcocystis medusiforms* and *S. tenella*, the last being the most pathogenic to sheep (Dubey *et al.*, 1989a and Dubey *et al.*, 1989b). Sheep are generally infected by *S. gigantea* when ingesting food or water contaminated with sporocysts that are shed by felids in environment. Macroscopic cysts in muscles are important for carcass meat trimmings and subsequently in heavy infection of carcasses are condemned, and so cause lower nutritional value of meat and economic losses for flock owners. However, microscopic cysts are important with regard to clinical signs such as anorexia, fever, decreased weight gain, anemia and abortions in ewes (Pescador *et al.*, 2007). These damages and pathological signs are more commonly associated with *Sarcocystis arieticanis* and *S. tenella*. Asia represents the highest occurrence, with 96.9% in Mongolia (Fukuyo *et al.*, 2002), 47.3% (Özkayhan *et al.*, 2007) to 86.5% in Turkey (Beyazit *et al.*, 2007), 33.9% (Daryani *et al.*, 2006) to 100% in Iran (Hamidinejat *et al.*, 2014), 93% in Ethiopia (Woldemeskel & Gebreab, 1996), 91.7% in Romania (Adriana *et al.*, 2008), and 84% in the United States (Dubey *et al.*, 1988). Generally, diagnosis of *Sarcocystis* spp is performed by different methods, including checking symptoms, sampling of carcass, squeezing out and enzymatic digestion in order to perform

microscopic and histologic examination, observation of carcass in macroscopic cases, serologic diagnosis, electron microscopic, and recent molecular techniques. Application and usage of each of them is varied based on direction and purpose. For example, serological methods are used for screening and wide epidemiological studies, but this method confers some limitations in diagnosis of parasite species and its strains.

Therefore, molecular diagnosis is highly sensitive to detection and differentiating the parasites. The most important of the serological methods for diagnosis of *Sarcocystis* is the immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) technique, but one limitation is the possibility of cross reaction with other heterologous species.

The development of molecular methods for the sensitive diagnosis, genotyping, and sub genotyping over the last few years has allowed the identification of sources of oocyst contamination and routes of transmission in both outbreak and non-outbreak situations to be examined (Xiao, L. 2010). SSU RNA gene is highly specific for the diagnosis of *Sarcocystis* genus and for determining species, and using appropriate restriction enzyme creates unique species-specific fragments.

The specificity of the 18S rRNA was previously confirmed by Tenter *et al.*, 1992 and Tenter *et al.*, 1994, Ellis *et al.* (1995), Guo and Johnson (1995), Jeffries *et al.* (1997) and Yang *et al.* (2001) and was considered as a powerful tool for species-specific differentiation of the ovine *Sarcocystis* species (*S. tenella*, *S. capracanis*, *S. arieticanis* and *S. gigantea*), *N. caninum* and *T. gondii*.

This PCR detects more copies than 18S rRNA. Thus, this report demonstrates the presence of *S. gigantea* in commercial sheep bred extensively in important economical regions from the north of Iran for sheep flock breeding, demonstrating the importance of the molecular techniques as an epidemiological and diagnostic tool to determine the agent(s) involved in the infection or co-infection with other pathogens such as *T. gondii*.

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