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ORIGINAL ARTICLE

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Molecular detection of *Sarcocystis* species in slaughtered sheep by PCR–RFLP from south-western of Iran

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Abstract *Sarcocystis* spp. are the cyst forming protozoan parasites that prevalent in livestock all around the world. In the presented work, we examined 40 macroscopic and 40 microscopic sarcocysts from Khouzestan and Lorestan provinces, south-western Iran, utilizing PCR–RFLP based on amplification of 18S rRNA gene. Using *Ava*I, *Hin*dII, *Taq*I and *Eco*RI restriction enzymes the results represented *Sarcocystis gigantea* in both macroscopic and microscopic cysts. This result supports the importance of molecular investigations on characterization of *Sarcocystis* species when we aimed to assess the reliable control and preventive programs.

Keywords *Sarcocystis* spp. · PCR–RFLP · 18S rRNA · Sheep · Iran

Introduction

Sarcocystosis is a zoonotic disease in domestic animals caused by *Sarcocystis* spp. (Apicomplexa: Sporozoa), a cyst forming coccidian parasite with obligatory two-host life cycle involving carnivorous as definitive hosts and herbivorous or omnivorous as intermediate hosts. Each intermediate and definitive host may harbour more than one *Sarcocystis* species (Dubey et al. 1989aa; Dubey and Lindsay 2006).

The parasites are most prevalent in livestock animals including sheep all around the world (Dubey et al. 1989b). Sheep become infected with the parasite via ingesting sporocysts or sometimes sporulated oocysts existed in the food or water (Dubey and Lindsay 2006). Four species of *Sarcocystis*, known as: *Sarcocystis tenella*, *Sarcocystis arieticanis*, *S. gigantea* and *S. medosiformis* have been documented and described in the Sheep. Of those *Sarcocystis* species that infect sheep, *Sarcocystis gigantea* and *S. medusiformis* are transmitted by felids and are consider non-pathogenic, whereas microscopic cyst forming species, *S. tenella* and *S. arieticanis*, which are transmitted by canids can cause devastating sarcocystosis that may be result in abortion or death depends on the dose of ingested sporocysts and the immune status of the host (Buxton 1998; Dubey et al. 1989b; Dubey and Lindsay 2006).

The conventional tools for species diagnosis of *Sarcocystis* spp. were based on transmission electron microscopy, structure of the cyst wall in the striated muscles of the intermediate host or information about the lifecycle of the parasite (Jehle et al. 2009). However, because of showing the morphologic variations in these procedures they are not exactly reliable at the species-specific identification. On the other hand, electron microscopy is not a choice for wide and extensive detective studies (McManus and Bowles 1996).

In recent times, various molecular techniques such as PCR and its variants based on sequence changes have been used regarding the sensitivity and rapidity to determine genetic diversity among many parasites, phylogenetic and

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taxonomic studies and in epidemiological mapping (Erlich et al. 1991; Gonzalez et al. 2006; Maurer 2011).

Currently, PCR-restriction fragment length polymorphism (PCR–RFLP) based on variable regions of the small subunit ribosomal RNA sequences is considered and used widely as a rapid, inexpensive and accurate molecular approach to discriminate different protozoa as well as *Sarcocystis* spp. (Ellis et al. 1995; Heckeroth and Tenter 1999a; Heckeroth and Tenter 1999b; Motamedi et al. 2011). As sheep are rearing all around Iran and are the strategic animal in livestock industry, so, the aim of this study was to determine the *Sarcocystis* species of sheep in two economically important provinces, Khouzestan and Lorestan, by PCR–RFLP in basis of amplification of 18S rRNA gene.

Materials and methods

Samples collection

Totally 40 macroscopic cysts (20 from Khouzestan province and 20 from Lorestan province main slaughterhouses) from sheep muscles were collected. Also, at least 50 g from esophagus, diaphragm, intra-costal, abdomen and biceps were sampled for microscopic investigations. The samples were kept at −20 °C until examination. The sampling discontinued when the positive specimens for microscopic cysts reached to 40 specimens (20 from Ahvaz and 20 from Khorammabad).

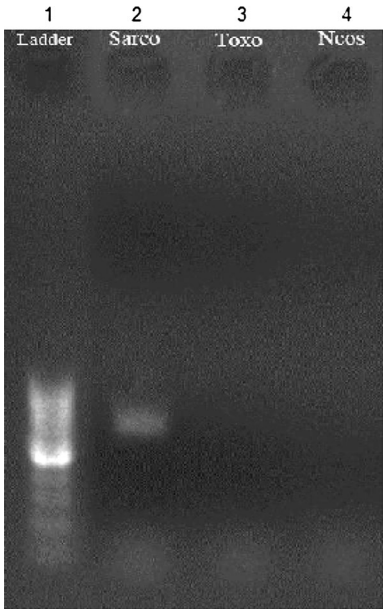

Fig. 1 PCR analysis of 18S rRNA gene with *T. gondii*, *N. caninum* and *Sarcocystis* sp. Revealed only 690 bp band for *Sarcocystis* sp. (*1*) 100 bp ladder, (*2*) Sheep *Sarcocystis* bradyzoite, (*3*) *T. gondii* tachyzoite, (*4*) *N. caninum* tachyzoite

Digestion method

The method of Dubey et al. (1989a) with some modifications was used for digestion of muscles as described below:

100 g of mixture of collected muscles were minced and digested for 30 min at 37 °C in 100 ml of digestion medium containing 1.3 g of pepsin (Merck), 3.5 ml, and 2.5 g NaCl in 500 ml of distilled water. After digestion, the mixture were centrifuged for 3 min at 3500×*g* and the sediment was then stained with giemsa and examined by optical microscope at 400× magnification for detecting bradyzoites. After that, the sediments containing bradyzoites washed two times with normal saline and TE solution respectively and preserved at −20 °C until examination.

DNA extraction

DNA was extracted using the genomic DNA extraction Kit (Cinnagen, Iran) according to manufacturer instructions. Next, the extracted DNA was stored at −20 °C until PCR has performed.

PCR amplification and RFLP

Species determination was carried out by PCR–RFLP according to amplification of 18S rRNA gene. PCR protocol and primer selection were adopted according to the previously described by Paikari et al. 2008 with some modification.

Briefly, 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, 3.0 mM $MgCl_2$, 5.0 µl 10× 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. 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. To verify the results, 10 µl of each PCR product was electrophoresed in a 1 % agarose gel, stained with safe stain and visualized on a UV transilluminator. The PCR products were identified by size using a 100 bp ladder (Fermentas). The expected PCR product had a length of 609 bp.

To determine the possibility of the cross reaction with related protozoans, *Neospora caninum* and *Toxoplasma gondii*, the whole tachyzoites of these two parasites were

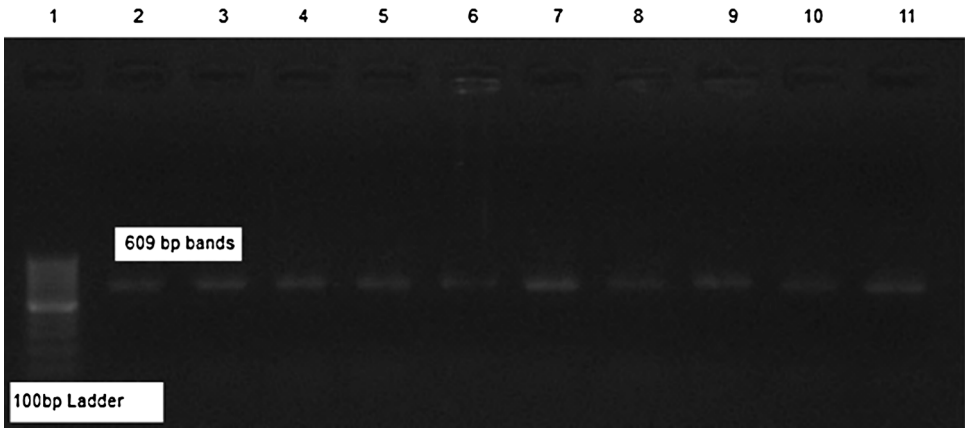

Fig. 2 PCR analysis of different isolates of sheep micro and macro sarcocysts (2–11)

also analyzed by mentioned primers. The amplified products were analyzed with RFLP using *Ava*I, *Hin*dII, *Taq*I and *Eco*RI restriction enzymes. Digested PCR products were resolved on a 1.5 % agarose gel and visualized with safe stain under ultraviolet light.

Results

PCR amplification of *T. gondii* and *N. caninum* tachyzoites with mentioned primers revealed no electrophoretic bands (Fig. 1) but, in the same situation our isolates demonstrated a *Sarcocystis* specific 609 bp band (Figs. 1 and 2).

Gel electrophoresis of the PCR–RFLP by amplification of 18S rRNA gene from all isolates of micro and macro sarcocysts showed that restriction with *TaqI* enzyme produced 270 and 339 bp fragments and also, *HindII* produced 198 and 411 bp fragments (Figs. 3 and 4). These results represent the *S. gigantea*.

Discussion

Thus far, four species of *Sarcocystis* have detected in sheep including *S. tenella, S. arieticanis* which transmitted by the felids and *S. gigantea* and *S. medusiformis* transmitted by canids (Dubey and Lindsay 2006; Adriana et al. 2008). Among these species *S. tenella* and *S. arieticanis* produce microscopic cysts and have pathogenic subsequences as an acute disease presented by abortion, fever, anemia and anorexia in early period of infection and then some chronic disorders maybe develop (Tenter 1995; Pescador et al. 2007). On the other side, macrocyst forming species, *S. gigantea* and *S. medusiformis* are considered nonpathogenic, but they can affect the meat quality and marketing and cause economic loss. Sheep maybe infected with these four species simultaneously (Tenter 1995; Dubey and Lindsay 2006).

At yet, various conventional procedures such as trichinoscopy, staining with methylene blue, dob-smear, digestion and histology have been employed for diagnosis of sarcocystosis in meat samples. These methods are genus-specific and just performable on slaughtered carcasses (Williams et al. 1990; Verhasselt et al. 1992; Holmdahl et al. 1993).

On the other hand, some serological assays including enzyme linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT), based on bradyzoites derived from sarcocysts have been assessed for serological diagnosis of sarcocystosis in sheep as well as some other animals (Uggla and Buxton 1990; Moré et al. 2008; Moré et al. 2010). Since, the bradyzoites of different *Sarcocystis* spp. have very antigenic similarities and therefore they have considerable cross reactions with other *Sarcocystis* spp. and other related parasites (Moré et al. 2010), it is obvious that these serological methods have serious limitations for species diagnosis as well as the genus determination.

In recent years, molecular diagnostic techniques have been assessed for specific determination of *Sarcocystis* spp. (Yang and Zuo 2000; Yang et al. 2001). Among various genomic targets, the highly conserved 18S ribosomal subunit is used widely for species-specific detection of different protozoa as well as *Sarcocystis* spp. due to presentation of highly variable regions (Yang and Zuo 2000). On the other hand, many authors confirmed the 18S rRNA for firmly species-specific discriminating of sheep sarcocysts (Tenter et al. 1992, 1994; Ellis et al. 1995; Yang et al. 2001). Thus, the aim of our study was to determine the *Sarcocystis* species of sheep in Khouzestan and Lorestan provinces using PCR–RFLP in basis of amplification of18S rRNA. The sampling was from esophagus, diaphragm, intra-costal, abdomen and leg muscles of sheep. Reported by Buxton (1998) preferred organs for *Sarcocystis* spp. in the intermediate hosts are heart, diaphragm, and skeletal muscles which the parasite can remain in these sites all long life of the intermediate hosts.

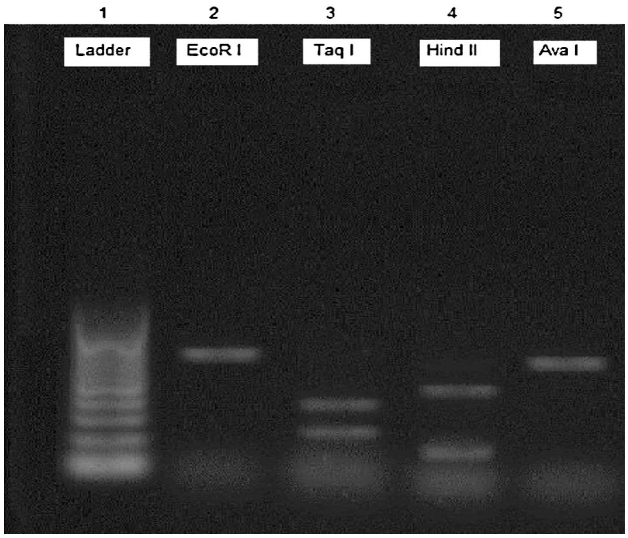

Fig. 3 PCR–RFLP analysis of PCR products of the 18S rRNA gene from isolated microcyst of *Sarcocystis* sp. from examined sheep digested by *Eco*RI, *Taq*I, *Hind*II and *Ava*I restriction enzymes

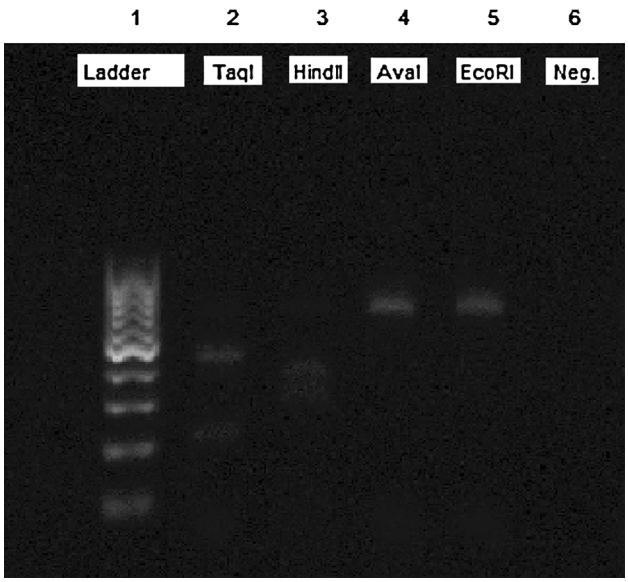

Fig. 4 PCR–RFLP analysis of PCR products of the 18S rRNA gene from isolated macrocyst of *Sarcocystis* sp. from examined sheep digested by *Eco*RI, *Taq*I, *Hind*II and *Ava*I restriction enzymes

The results of our study revealed that only *S. gigantea* was distinguished in Khouzestan and Lorestan sheep and multiple infections with *Sarcocystis* spp. was not present. (Paikari et al. 2008) reported this species for the first time in Qazvin city, Iran but they find also *S. arieticanis* in their study.

Also, in this work we did not detect any cross reactions with *T. gondii* and *N. caninum*. Homan et al. (2000) also reported no cross reaction between *T. gondii* and *Sarcocystis* spp. or *N. caninum* in PCR results.

From the other point of view, since our work demonstrates only the presence of *S. gigantea*, we can conclude that probably acute clinical sarcocystosis which is related to *S. tenella* and *S. arieticanis* is not considerably important in differential diagnosis of sheep disorders such as abortion for example induce by toxoplasmosis in the two different and economically important areas of Iran and because of the definitive hosts of *S. gigantean* are the cats, so we must concentrate on this animal in control and prevention programs of *Sarcocystis* infection in sheep. Although, in order to prohibit of probable transmission of other *Sarcocystis* species access of dogs to sheep herds must be denied (Adriana et al. 2008). These conclusions clearly reveal and support the importance of molecular investigations to identify the etiology of different diseases, especially when these methods are more accurate and economically superior in compare with other methods (Pescador et al. 2007).

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