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# Identification of *Sarcocystis tenella* Isolated from Sheep in Tabriz Abattoir by Parasitological and PCR-RFLP Methods

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***Sarcocystosis* is caused by species of *Sarcocystis*, an intracellular protozoan parasite in the phylum Apicomplexa. These parasites have an indirect life cycle, cycling between a definitive and an intermediate host. Intestinal infections occur in the definitive host, and tissue invasion is seen in the intermediate host. In this study, heart and diaphragm muscles of 60 sheep were collected from Tabriz abattoir and assessed for the presence of *S. tenella* using methods of impression smear, tissue digestion, and PCR-RFLP techniques. Microscopic cysts was identified by preparation of direct tissue impression smears from samples and staining them by Giemsa stain and digestion of samples by pepsin and finally centrifugation and preparation of smear from the sediment and staining them with Giemsa stain. We found the microscopic cysts in 40 % of impression smears and 100% of tissue digestions. We observed *S. tenella* in 70% of muscle samples by using of PCR-RFLP method. This study showed that tissue digestion method is more sensitive method for Sarcocystis. PCR-RFLP technique by use of specific primers and TAG1 enzymes is shown to be an easy and rapid method for identification of species.**

**Key words:** Sarcocystis, Pepsin digestion, PCR- RFLP, Iran.

Various species of Sarcocystis cause sarcocystosis or sarcosporidiosis the genus that infects human and animals[1]. Is composed of about 130 species of heterogeneous cyst forming coccidian. The members of this genus have different life cycle, consisting of merogony, gamogony and sporogony, and pathogenicity and are obligatorily intracellular protozoa[2]. They need to both intermediate and definitive hosts for continuing of the life cycle[3]. Sheep are considered as important intermediate hosts of four *Sarcocystis* species including *Sarcocystis tenella* (synonym *Sarcocystis ovicanis*) and *Sarcocystis gigantea* (synonym *Sarcocystis ovifelis*) with world-wide distribution, *Sarcocystis arieticanis* in USA, Europe, New Zealand and Australia and *Sarcocystis medusiformis* that have been reported only from Australia and New Zealand. Merogony and cyst formation are the ways of multiplication of the parasite in the intermediate host. Gamogony

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and sporogony are take place in the definitive host[4]. Sarcocystis species usually infects only one species of intermediate host. In other word, a given species infecting domestic animals uses either felids or canids, but not both, as definitive host[5]. *S. tenella* and *S. arieticanis* are pathogenic and are transmitted by canids. They develop microscopically visible cysts. *S. gigantea* and *S. medusiformis* that develop macroscopically visible cysts and are transmitted by felids are non-pathogenic[3]. *S. tenella* causes causing significant losses in the livestock industry. Death of the animal or abortion of pregnant ewes during acute sarcocystiosis has been frequently reported. It causes widespread enzootic muscle parasitosis, and reduces weight gain, milk, and wool production through chronic sarcocystiosis[4]. Lambs neurological disease has been reported[6,7]. Human Sarcocystosis may be diarrhea, episode of vomiting, cramping abdominal pain and may result in perverse illness in-patients with acquired immunodeficiency syndrome or other immunosuppressive diseases[8]. Ingestion of sporocysts of *S. tenella* through contaminated food or water can infect the sheep. *S.tenella* similar to two other protozoans, *Toxoplasma Gondi,* and *Neospora caninum* can cause encephalitis in sheep[9]. Despite the High prevalence of Sarcocystosis in different livestock In Iran[10,11], a few reports about to identify parasite by molecular methods have been published, and there is not any report on identification of the parasite from animals from East Azerbaijan province. This study was designed and carried out to identify and to reveal the importance of sarcocystosis in the province.

## MATERIALS AND METHODS

Muscles of heart and diaphragm of 60 sheep slaughtered in Tabriz abattoir were assessed for Sarcocystis infection. Microscopic examinations were performed in parasitology laboratory sing at least 70 g of heart and diaphragm muscles. Small pieces of fresh muscle were compressed between two glass slides and impression smear prepared was stained by Giemsa stain and assessed for bradyzoite with optical microscope at 400X.Digestion of muscles was performed with some changes of method previously described[4]. 50 mg of collected muscles were digested in 100 ml of digestion medium containing 1.3 g of pepsin (Merck) 3.5 ml HCl and 2.5 g NaCl in 500 ml of distilled water for 30 min at 37°C. The mixture were centrifuged at 2500x g for 5 min after digestion and the sediment was stained with giemsa and assessed by light microscope at 400x magnification for detection of bradyzoites. Bioneer DNA easy commercially available kits were used for DNA extraction according to the manufacturer's instructions. For amplifying, the gene (18S rRNA) of *S. tenella* through method of Polymerase Chain Reaction (PCR) specific primers was used including: Forward primer ACGGCGAAACTGCGAATGGCT and reverse primer CGCGCCTGCTGCCTTCCTTA yielding a product of expected length 398 bp using standard conditions. Amplification condition was as follows: initial denaturation 95º C, 5 min; 94ºC , 45 s, 57.8ºC, 1min and 72ºC , 1 min and final extension 72ºC, 7 min. Visualization of the PCR products was performed using 1.5 % agarose gel electrophoresis with ethidium bromide in a UV transilluminator. The amplified PCR products were digested with TAG1 restriction enzyme through PCR-RFLP method for identification of Sarcocystis species. Briefly, a total of 25-µl reaction mixture containing 10–15 µl PCR product, 1 units of restriction enzyme, 2 µl of appropriate buffer, 2 µl of BSA, and 10 µl of water was used. According to the manufacturer's recommendations, the visualization of the products was performed using 3% agarose gel electrophoresis with ethidium bromide in a UV transilluminator. Commercial 100-bp DNA ladder was used for estimating the size of the resulting DNA fragments. The PCR –RFLP yielded an amplicon of approximate length 284 bp and 59 bp.

## RESULTS

Bradyzoite of Sarcocystis observed in 100% of examined muscles of 60 sheep through peptic digestion. Direct tissue smear preparation showed 40% of infection in sheep (45 of 60). After amplification of related part of 18S rRNA gene, we noted the expected PCR product size (with length of approximately 398 bp) in 70% of microscopic positive samples (Fig 1). Pattern identical with *S. tenella* was produced from PCR products after digestion with restriction enzyme TAG1in RFLP

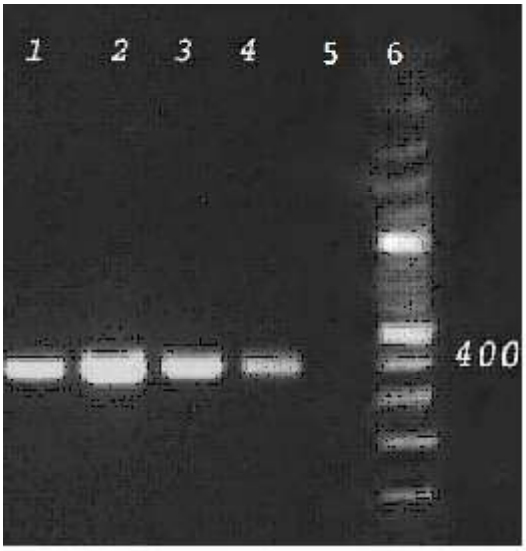


**Fig. 1.** Ethidium bromide–stained 1/5% agarose gel showing amplification of a 398-bp product for *s. tenella* isolated from sheep. Lanes 1–4, s. *tenella*-positive samples; lane 5, uninfected tissue (negative control); lane 6, DNA size marker (100 bp Ladder)

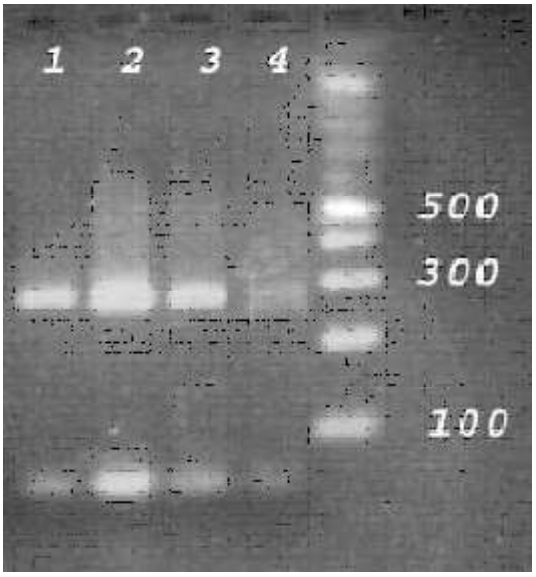


**Fig. 2.** PCR-RFLP analysis of Sarcocystis isolated from Iranian sheep showing species specific fragment sizes of *S. tenella* after digestion with TAG 1 (approximately 284 and 59 bp)

method that is two fragments of approximately 284 and 59 bp (Fig 2).

## DISCUSSION

Naturally, different pathogen and nonpathogenic species of *Sarcocystis* may infect the sheep at the same time. *Sarcocystis* sporocysts can be found in fecal samples of canids. Traditional methods of diagnosis have low specificity and sensitivity for diagnosing of abortion and acute disease caused by *S. tenella* and *S. arieticanis*.[3]. As, Dogs, the definitive host, have free-access to the flock in farms or arthropods as mechanical vectors of Sarcocystis sporocysts excreted in feces, easily infect with the parasite[12]. Heart, diaphragm, and skeletal muscles of intermediate hosts are mainly, infected with Sarcocystis. Infection can persist in the host lifelong[13]. Various conditions including involvement of the variety of definitive hosts in transmission of *Sarcocystis*, shedding of large number of sporocysts (as infective form) for many months, resistance of oocysts or sporocysts to environmental conditions and role of invertebrate hosts in transmission of infection facilitate high prevalence of Sarcocystiasis[14]. Morphological characterization of cyst wall and sporocysts of sarcocysts using light microscopy is routine diagnostic method, but transmission and scanning electron microscopy also is used for identification of the parasite species in domestic animals. Palisade-like protrusions, consistent with those of *S. tenella*, are seen in transmission electron micrographs of the primary wall of mature sarcocysts from the heart, tongue, and skeletal muscles[15].There are several reports on the prevalence of Sarcocystis in livestock from different regions of Iran using microscopic examination of fresh tissue. High prevalence of Sarcocystis in sheep reported from various parts of Iran[16-19]. An increased number of sarcocystosis in water buffalo from Khuzestan, western Iran and increase distribution of the disease has been shown through recent epidemiological data[20]. Because of some restrictions of microscopic diagnosis of Sarcocystis different location and developmental stage and criteria of the parasitized cell, confirming of identification of Sarcocystis species with more

sensitive and specific methods may be needed[21,22]. Isolation of Sarcocystis from the slaughtered sheep using PCR-RFLP of 18SrDNA gene and identification of macroscopic cysts of Sarcocystis gigantean and microscopic cysts of Sarcocystis arieticanis has been reported from Iran[23]. Molecular diagnosis is considered as a reliable method for species-specific differentiation of the four ovine Sarcocystis species. The high sensitivity and specificity of the PCR-RFLP method with amplification of 18S rRNA gene has been confirmed frequently[24].In this study, we showed the presence of S.tenella in 100 %of sheep isolated from Tabriz abattoir by technique of by peptic digestion and we observed *S. tenella* in 70% of muscle samples by using of PCR-RFLP method. Such a difference between the methods for diagnosing the parasite has been shown previously[25,26]. Shows the importance of the molecular techniques as a reliable epidemiological and diagnostic tool to determine the Sarcocystis species. Additionally, in comparison with DNA sequencing the approach is cost effective and rapid. Pepsin or trypsin digestion, muscle compress and histopathological methods are several diagnostic methods used for the identification of sarcocystosis[27]. There are different reports on the prevalence of *S. tenella* in sheep worldwide. It has occurred in 96.9% in Mongolia[28], 93% in Ethiopia, 91.7% in Romania[12], 84% in United States, 47.3% (29) to 86.5% in Turkey[27], and 33.9% in Iran[13]. In present study both microscopically and molecular examination (PCR-RFLP) used for diagnosis of *Sarcocystis tenella* infection in sheep at Tabriz abattoir. Totally, in our study high frequency of *Sarcocystis,* infection was observed in sheep by microscopically examination and *S. tenella* infection was confirmed in muscles (diaphragm/heart) of sheep by using of PCR-RFLP method. The high frequency of microscopic sarcocystis infection in sheep will be associated whit the fact the health system should provide an effective and comprehensive program to manage and control of zoonotic diseases and to decrease social and economical burden of this problem.

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