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Prevalence of Ovine and Bovine Theileriosis in Domestic Ruminants based on 18s rRNA Gene and Microscopic Techniques in Qazvin Province, Iran

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A total of 165 blood samples were collected in k2EDTA-containing tubes and thin and thick smears of domestic ruminants were prepared. Direct sequencing of polymerase chain reaction for detection of *Theileria* spp. using specific oligonucleotide primers was performed on 18s rRNA gene sequence of the parasite in Qazvin province. Of the 165 field samples obtained from domestic ruminants in the study area tested, 9.69% (16) were positive for the presence of *Theileria* spp. by PCR and 2.42% by microscopic examination for *Theileria* spp. in field-collected. Out of 165 positive blood samples, 4.24% were positive for *Theileria annulata* and 5.45% were positive for *Theileria ovis* by PCR. The lowest detection limit of the PCR was two parasites per ml of infected blood, which corresponds with a parasitemia of 0.00004%. Results of the molecular study revealed that *Theileria ovis* is widespread in sheep and its prevalence was higher than *Theileria annulata*.

Key Words: *Theileria annulata*, *Theileri ovis*, domestic ruminants, PCR.

Theileria spp., the causative agents of theileriosis, is hemoprotozoan parasites of domestic, and wild mammals (Kocan and Kocan, 1991). The correct identification of these organisms is crucial for understanding their epidemiology (Oura *et al*, 2004). *Theileria lestoquardi* and *Theileria ovis* are two notorious species, for ovine and *Theileria annulata* for bovine theileriosis in Iran (Shayan *et al*, 2008; Heidarpour Bami *et al*, 2010; Azizi *et al*, 2008; Hashemi-Fesharaki, 1997). According to clinical signs and morphological observations, *T. ovis* is widespread all over the country (Hashemi-Fesharaki, 1997). However, the epidemiological aspects of ovine and bovine theileriosis in Iran haven't completely understood (Haddadzadeh *et al*, 2004). There are several methods for detecting and identifying these hemoparasites including microscopic examinations of Giemsa-stained thin and thick blood smears, tick salivary gland staining, serological tests and clinical symptoms (Schnittger *et al*, 2004; Jongejan, 1983). In contrast to these usual methods, the use of molecular techniques would allow direct, rapid, specific and simultaneous detection of *Theileria*

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parasites (Schnittger *et al*, 2004; Jongejan, 1983). The use of such methods has prepared comprehensive information of epizootiology in quantitative terms, but as sequencing of isolates is rarely undertaken, qualitative aspects remain less well understood. In the present study, 18 S rRNA genes of *Theileria* sp. isolates from domestic ruminants were sequenced to confirm and determine prevalence and distribution of various *Theileria* species involved in ovine and bovine theileriosis in Qazvin province of Iran. Nuclear ribosomal rRNA genes have been shown to provide suitable targets to assist in the identification of *Theileria* species (Katzer *et al*, 1998; Chae *et al*, 1998; Allsopp and Allsopp, 2006).

MATERIALS AND METHODS

Field of the study area

The survey was done in Qazvin province, one of the 31 provinces of Iran, which is located in north-west of the central plateau of Iran (Fig. 1). Its center is the city of Qazvin and has five counties including Qazvin, Abyek, Boeen- Zahra, Alborz, and Takistan (Fig. 1). According to the census of the veterinary organization of Qazvin province in 2009, it has 164,000 cattle, 874,000 sheep, and 126,000 goats. Most of the livestock are of a domestic race and low percentages are mixed breeds, including Holstein (Iranian Veterinary Organization, unpublished data). Sheep husbandry is one of the most economically important jobs in this province. Sheep and goats are kept together and the sheep outnumbered goats in each flock (Shemshad *et al*, 2010).

Collection of blood samples

In this study 66 flocks were visited from April to September 2010 to detect the presence of various *Theileria* spp. A totally of 165 peripheral blood samples and thin and thick smears were collected monthly in k2EDTA from randomly selected sheep, goats and cows in Qazvin Province. The selected animals were clinically investigated and blood smears were prepared from the marginal ear veins. The smears were air-dried, fixed in methanol in the field and were sent to the laboratory in a cold box kept at 4°C.

Examination of blood smears

The smears stained in 10% Giemsa solution for 30 minutes and were observed with an oil immersion lens with a magnification of 1000 under light microscopy to detect *Theileria* piroplasms. The full length of the intraerythrocytic mature *Theileria* organism was measured by a graded ocular microscope with a magnification of 1000. Parasitemia was calculated by counting the number of infected red blood cells by examination of 100 microscopic fields (approximately 100,000 cells). The number of infected cells was then expressed as a percentage.

DNA Extraction and PCR amplification

Genomic DNA was extracted using a whole blood DNA extraction kit (G-spin™, iNtRON Biotechnology, Korea) according to the manufacturer's instructions. Briefly, cells were first lysed in 180 µl lysis and incubated at 70°C. After addition of 300 µl Bindings buffer and centrifuging for 1min at 13,000 rpm, 500 washing buffer was added and after vortexing, centrifuged twice for 1 min. Finally, DNA was eluted from the carrier with Elution buffer and was stored at -20°C until subsequent analysis. In some cases, the DNA isolated with the Phenol/Chloroform method. One pair of screening primers was used to amplify the DNA of all *Theileria* spp. PCR amplification was performed with the forward strand primer P1: 52 cacagggaggtagtgacaag 32 and reverse strand primer P2: 52 aagaatttcacctatgacag 32 that was specific to 18S rRNA gene of both *Babesia* and *Theileria* species (Shayan *et al*, 2008) (Table 1).

Table 1. The sequences for primers used in PCR from hyper variable region V of 18S rRNA gene of *Babesia* spp. and *Theileria* spp.

PCR product (bp)	Name of primer	Name of primer	No.
389–402 (Babesia)	52 cacagggaggtagtgacaag 32	18S rRNA gene sense	P1
426–430 (Theileria)	52 aagaatttcacctatgacag 32	18S rRNA gene antisense	P2

Each 25 ml amplification reaction in iNtRON's Maxime PCR premix kit component contained *i-Taq*™ DNA polymerase (5U/ µl), dNTPs 2.5 mM each, Reaction buffer (10x) 1x, gel loading buffer 1x, 1 pmol/ µl each of P1 and P2 reverse primer derived from hyper variable region V4 of 18S rRNA (P1 and P2), 5 µl template DNA and 13 µl distilled water. The amplification conditions for the PCR initiated with denaturation at 95°C for 5 min, followed by 35-38 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 45 sec and terminated with a final extension at 72°C for 10 min and hold at 4°C until use. Negative (no template) and positive controls were always run at the same time. The specific PCR products of the primary PCR was 426–430 bp. Appropriately sized amplicons were confirmed by electrophoresis in ethidium bromide stained 2% agarose gels which were then viewed by UV transillumination. For differentiation of various *Theileria* species positive direct sequencing of specific PCR products of the 18S rRNA genes of *Theileria spp*., was sequenced and analyzed (SEQLAB Sequence Laboratories Göttingen, Germany). Forward primer was used for sequencing. A BLAST search was performed with the obtained sequences using the BLASTn algorithm and compared with sequences deposited in GenBank.

RESULTS

Blood samples were collected in k2EDTA from 102 sheep, 32 goats and 31cows, in 66 flocks in Qazvin province. Thin and thick smears were prepared from the ear veins of all randomly selected animals and their examination of domestic

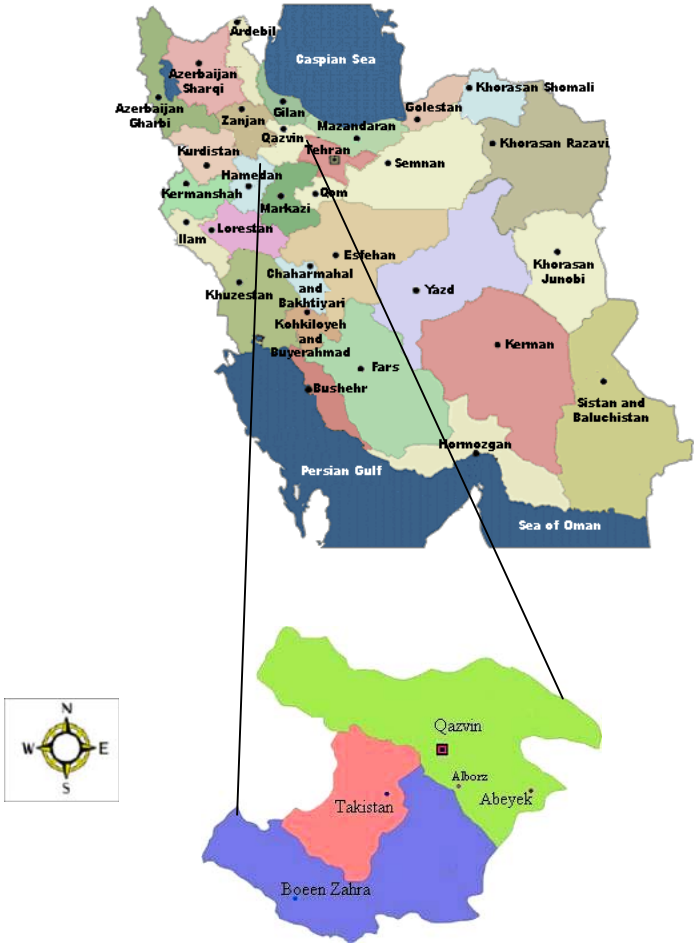

Fig. 1. Map of Iran and location of Qazvin province, Iran Meteorological organization 2010

ruminants showed 0.0004% (2.42) parasitemia and ring form and Koch blue body of the parasite were seen inside the red blood cells for *Theileria* spp. piroplasms (Fig. 2). DNA was extracted from all prepared peripheral blood of randomly selected ruminants using phenol/chloroform method, and G-spin™ -Kit. Amplification the DNA sample using P1/P2 primer set for PCR and analysis these PCR products on the 2% agarose gel electrophoresis, showed 16 out of 165 blood samples (9.69%) positive for *Theileria* sp. while this rate was only 4 (2.42%) in field-collected samples by microscopic examination Giemsa-stained blood smears for piroplasmic forms of *Theileria annulata* and *Theileria ovis*. Out of 165 positive blood samples, 4.24% (7) were positive for *T. annulata* and 5.45% (2 for goat blood and 7 for sheep blood) were positive for *T. ovis* by PCR.

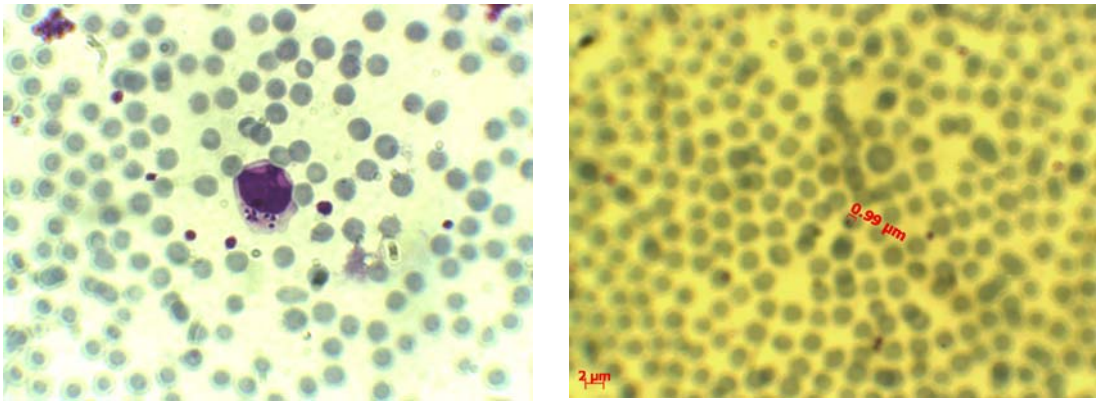

Fig. 2. Koch blue body and ring form of *Theileria ovis* in microscopic field by Giemsa staining method

The lowest detection limit of the PCR was two to three parasites per ml of infected blood, which corresponds with a parasitemia of 0.00004%. *Theileria*-specific 18s rRNA primers P1 and P2 were used as a positive control and amplified the expected fragment. Not only, all of the positive samples by thin and thick blood smears were also positive by PCR, but also they were positive for the slides that no piroplasm and schizont stage were seen by light microscopy (Fig. 3).

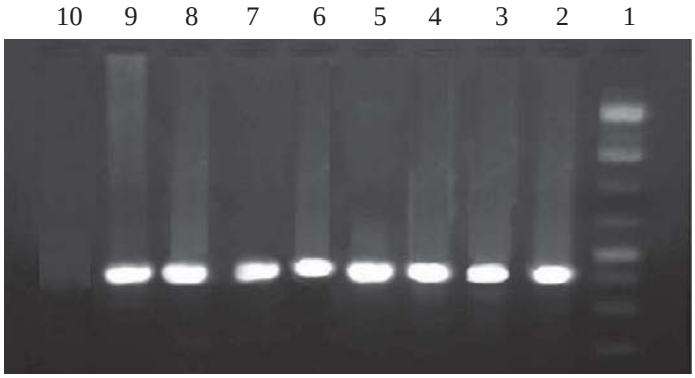

Fig. 3. Agarose-gel electrophoresis of amplication products obtained from *Theileria* spp. genomic DNA amplified using *Theileria*-specific primers (P1/P2) derived from the 18s rRNA gene. Lane 1: DNA size marker (1 kb Plus DNA Ladder, #SM1343, Fermentas), Lane 2: *T. ovis* (sheep), Lane 3: *T. ovis* (goat), Lane 4: *T. ovis* (goat), Lane 5: *T. ovis* (sheep), Lane 6: *T. annulata* (cattle), Lane 7: *T. annulata* (cattle), Lane 8: *T. annulata* (cattle), Lane 9: positive control, Lane 10: distilled water (negative control).

Having purified PCR product positive samples, all of the expected 388-395 bp fragments of 18S rRNA gene, were then sequenced and were compared with sequences of related genera and, confirmed all of them belong to the *Theileria* spp. The BLAST search about *Theileria ovis* revealed highest similarities (100%) with partial sequence data of *Theileria ovis* (FJ608733.1) (Italy), and about *Theileria annulata*, 99% similarity with a partial sequence of *Theileria annulata* (HM628582.1) (Iran). The nucleotide sequences of the 18S rRNA gene detected from domestic ruminants of Qazvin Province have been deposited in the GenBank database under the accession numbers: JN412658– JN412673.

DISCUSSION

Theileriosis is an intraerythrocytic disease, in infected domestic and wild ruminants (Durrani *et al*, 2011; Parthiban1 *et al*, 2010; Spitalska *et al*, 2004). It is one of the most important diseases that cause large economic losses in every involved country including Iran (Oliveira *et al*, 1995; Heidarpour Bami *et al*, 2010). However, a gap of information exists about the epidemiology of theileriosis in Iran. In the present study, the occurrence of various *Theileria* spp. infections in sheep, goat and cow Qazvin province was investigated using blood smear identification, and PCR-direct sequencing. In the present study, PCR on specific amplification of a 388-395 bp fragment of the hyper variable region V4 of 18s rRNA gene of the parasite showed 4.24% from the blood samples of the examined animals were positive for *T. annulata* and 5.45% were positive for *Theileria* ovis and confirmed by sequencing. Results of the molecular study showed that *T. ovis* has high prevalence in sheep and its prevalence was higher than *T. annulata*. The results were similar to those reported (Hashemi-Fesharaki, 1997) and revealed that *T. ovis* is distributed throughout the country.

The results obtained from field samples collected from sheep indicated that amplification of parasite DNA is more sensitive than detection by light microscopy. We found that the sensitivity of the examination was up to two piroplasms per ml of ovine blood. Only *Theileria ovis* was identified in sheep and goat flocks in the different regions. As most of the examined animals had no clinical signs so, it can be concluded that that subclinical infections were common, and couldn't be detected by microscopy examination.

The other reasons are that this method does not detect positive animals in the early phase of infection and the long-term carrier status, when the parasitemia is very low or destruction of piroplasmic forms in red blood cells as a consequence of deletion of typical shape of parasites in RBCs, the thickness, dirtiness or unsuitable blood smear staining occurs. Ruminants with clinical and sub clinical theileriosis are sources for tick infection since they carry piroplasms.

These results are similar to other investigations (Azizi et al, 2008; Oliveira *et al*, 1995; Kirvar *et al*, 2000; Kirvar *et al*, 1998; Aktas *et al*, 2002; Altay *et al*, 2005). It seems that animal movement plays a major role for spreading the disease into the northern part of the county and can cause theileriosis in sheep and cows. Many factors like climatic condition, susceptibility of breeds, distribution of vector system of breeding, vaccination, and strategy of prophylactic and treatment methods. The results show the necessarily of using molecular methods in determination of more accurate percentages of the infection rate and epidemiological condition of the animals and performing prevention and controlling programs in animals surviving piroplasm infection or carrier animals harboring a very low parasite level.

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