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Original paper

First molecular evidence of *Theileria lestoquardi* in small ruminants in northern Iran

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ABSTRACT. Ovine theileriosis as a critical agent in small ruminant production, can cause lethal infections. Different species of *Theileria* have been reported in various parts of the world, and each species causes different diseases in the host. This is the first molecular study to investigate the prevalence of ovine theileriosis and identify the dominant *Theileria* species in northern Iran. A number of 220 small ruminants, including sheep and goats, were randomly sampled from 22 flocks. Peripheral blood smears were stained by the Giemsa staining method. As well as for species identification, all samples were examined by PCR. From 220 samples, 160 and 60 were sheep and goat, respectively. By the Giemsa staining method, *Theileria* parasite was observed in 20 (9%) samples. But by polymerase chain reaction (PCR) method, 30 (13.6%) samples were positive for *Theileria* species. *Theileria lestoquardi* was the most common species found in these animals. The high prevalence of theileriosis in small ruminants demonstrates the emergence of ovine theileriosis in Mazandaran and Golestan provinces in northern Iran.

Keywords: theileriosis, prevalence, *Theileria lestoquardi*, small ruminants, PCR

Introduction

Ovine theileriosis is an important tick-borne disease of protozoan parasites of the Phylum Apicomplexa named *Theileria* [1,2]. *Theileria* are transmitted by ixodid ticks of several genera, including *Hyalomma*, *Amblyomma*, *Rhipicephalus*, and *Haemaphysalis* [3–5]. Theileriosis in wild and domestic animals causes high rates of mortality. This disease is prevalent in the tropical and subtropical areas including east, north and southern Africa, the Middle East and also some parts of Asia and Europe [6–8]. Many studies have been presented worldwide on bovine theileriosis but there is a lack of information about ovine theileriosis [9]. Different types of *Theileria* spp. have been reported in various parts of the world, and each species causes different diseases in the host. *Theileria lestoquardi* and the recently described *Theileria* spp. are considered to be extremely pathogenic, while *Theileria ovis* and *Theileria separata* are less pathogenic than *Theileria lestoquardi* [10]. Diarrhoea, anorexia, high fever, thinness, lethargy, swelling of the lymph nodes, eyelid swelling, increased pulse and panting, profuse lachrymation, anaemia, icterus, and death are all symptoms of theileriosis [11]. Unfortunately, the mechanism of the pathogenesis of malignant ovine theileriosis is not well known yet [11]. Identification of *Theileria* spp. is based on different techniques. The methods conventionally used to detect and recognize these hemoparasites consist of microscopic methods. In addition, serological methods are used in causal subclinical infections, but species differentiation is

Table 1. The sequences of specific primers used to the detection of *Theileria* genus in the studied samples [15]

Primers name	Nucleotide sequences	PCR product (bp)
P1 (forward)	5' CACAGGGAGGTAGTGACAAG 3'	426–430
P2 (reverse)	5' AAGAATTTCACCTATGACAG 3'	

difficult. The PCR technique has become a suitable method for diagnosis of ovine *Theileria* spp. in epidemiological studies, because this technique is more sensitive and specific than other predictable methods [12,13]. Although different species of *Theileria* parasite have been reported from different parts of Iran, the prevalence of theileriosis and its dominant species in some areas is not yet available. Due to the lack of any data about the occurrence of ovine theileriosis in Mazandaran and Golestan Provinces, northern Iran, this study was carried out, for the first time, to determine the prevalence of *Theileria* spp. and characterization of the parasite in domestic small ruminants, in these areas using microscopic examination and molecular technique.

Materials and Methods

Study area and sample collection

Twenty-two herds in Mazandaran and Golestan provinces located on the southern coasts of the Caspian Sea, northern Iran, in the summer of 2013 were selected for this study. Ten animals with pale or hyperthermic mucous membranes were randomly selected from each herd and peripheral blood smears were prepared from their ears. Finally, 220 thin and thick blood smears were collected from 220 sheep and goat suspected of theileriosis (100 from Mazandaran province and 120 from Golestan province) and transferred to the parasitology laboratory of Mazandaran University of Medical Sciences.

Peripheral blood smear

The Giemsa staining method was used for microscopic examination of peripheral blood smears for the presence of *Theileria*. Morphological parameters of the parasite such as the shape and location of each infected red blood cell are considered for accurate differentiation [14].

DNA extraction

To extract DNA from peripheral blood on the slide, initially, blood smears fixed were shaved with a scalpel and transferred to encoded micro tubes. DNA extraction was performed by the phenol chloroform isoamyl alcohol method. Blood samples were dissolved in lysis buffer and then 20 µl protainase k (10 mg/ml) was additional. After that to digest the proteins, the samples were incubated at 56°C for 2 hours. The mixture was then mixed with phenol chloroform and centrifuged at 13,400 rpm for 15 minutes. Supernatant was transferred to a new micro tube and 2.5 times the sample volume was added to that 96% ethanol. Then the samples were placed at –20°C for 45 minutes and then centrifuged at 13,400 rpm for 15 minutes. Washing was performed with 70% ethanol and finally extracted DNA air-dried, liquefied in Tris-EDTA (TE) buffer at 55°C, and finally kept at –20 degrees until use, finally, the amount of extracted DNA was determined by the Nano drop.

PCR assay

The genus of *Theileria* was identified by using specific primers derived from hyper variable region of 18SrRNA with a fragment length of 426–430 bp listed in table 1.

On the other hand, there is a difference of approximately 30 bp during PCR products of these two parasites, which can be easily detected by 1.5% agarose gel [15].

The PCR reaction with a final volume of 25 microliters (µl), including 12.5 µl master mix, 0.5 µl

Table 2. The sequence specific primers used to the detection of *T. lestoquardi* [16]

Primers name	Nucleotide sequences	PCR product
Theileria Le-sense	5' GTTACTCTCACTTCATGTGAG 3'	669 bp
Theileria Le-antisense	5' GGAGAACTTGTCGACAGCTGG 3'	

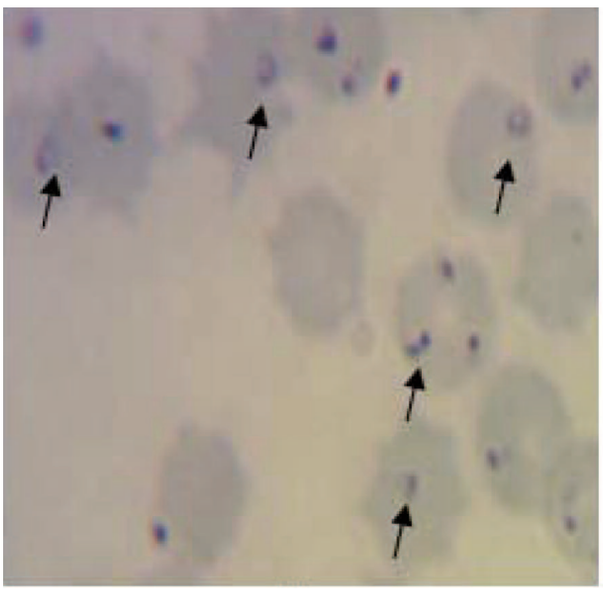

Figure 1. Various forms of *Theileria* in microscopic examination by Giemsa staining method (×1000 magnification)

of each primer, 5 µl of DNA template and 6.5 µl deionized water was performed by the Thermocycler (Bio-Rad). Also the temperature program used for the PCR reaction: 5 min at 95°C, 38 cycles of 94°C for 45 s, 56°C for 45 s, 72°C for 45 s and a final extension step at 72°C for 10 min. For visual detection by ultraviolet transillumination, we used 1.5% agarose gel electrophoresis with Sybr green stain.

Identification of Theileria lestoquardi

For PCR detection of *T. lestoquardi*, the final volume of the reaction was 25 µl considering 100 to 400 ng of DNA. The primer sequence used is given in table 2. Also, the temperature program used to perform the PCR reaction was: 95°C for 5 minutes to denature DNA, 35–38 cycles of 45 seconds at 54–58°C to denaturation, 45 seconds at 72°C for aneling and 45 seconds at 94°C to extension. PCR was accomplished with the additional extension phase at 72°C for 10 minutes [16]. Finally, 1.5% agarose gel and electrophoresis were used to confirm the amplification.

Ethics approval

This work was reviewed and approved by Research Ethical Committee at Mazandaran. Mazandaran University of Medical Sciences (IR.MAZUMS.REC.1391.136).

Results

Twenty (9%) out of 220 livestock were positive by microscopic method which is shown in the figure 1. While the results of the molecular study showed 30 cases (13.6%) infected with *Theileria* genus and also, by PCR technique, all positive cases were *Theileria lestoquardi* (Figs 2A and 2B).

The frequency of *Theileria* infection in Mazandaran and Golestan was 12% (12 of 100) and 15% (18 of 120), respectively.

Discussion

Theileria spp. infection is one of the most important protozoal diseases in domestic animals which can cause theileriosis [16]. Among the species of *Theileria*, one of the most virulent and the main cause of fatal disease is *T. lestoquardi* [17,18].

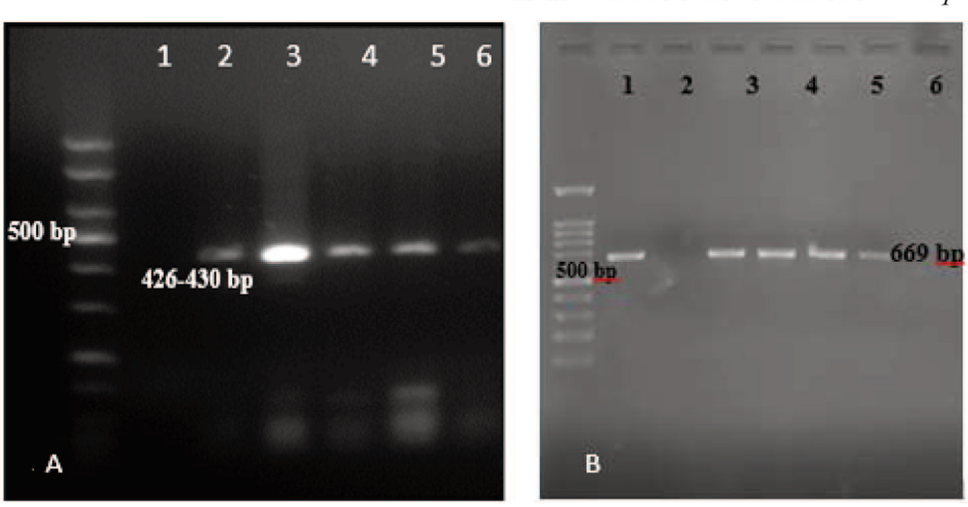

Figure 2. (A) PCR product on gel electrophoresis 1.5%. Lane 1: negative control, Lane 2: positive control (*Theileria* genus), Lane 3–6 samples; (B) PCR product on gel electrophoresis 1.5%. Lane 1: positive control (*Theileria lestoquardi*), Lane 2: negative control, Lane 3–6 infected ruminants. Ladder 100bp

Some *Theileria* species, including *Theileria lestoquardi*, *T. uilenbergi*, and *T. luwenshuni*, cause malignant *Theileria* in sheep, and in some cases have been observed in goats. Other species, including *T. ovis* and *T. separata*, have been found in small ruminants. According to [19], *T. lestoquardi* and *T. ovis* are important causes of malignant theileriosis in sheep and goats in Iran and are frequently reported from different parts of Iran, also other species such as *Theileria ovis* are less pathogenic and have less economic importance than *T. lestoquardi*. It is difficult to differentiate these two species (*T. lestoquardi* and *T. ovis*) based on the morphology of piroplasm and schizont phases, particularly in mixed infections. Exact differentiation between these parasites is crucial for finding and understanding the epidemiology of theileriosis [20]. To date, in Iran and other countries, detecting hemoprotozoan parasites such as *Theileria* spp. can be done by finding schizonts in peripheral blood smears and by some molecular methods such as standard PCR, semi-nested PCR, nested PCR, PCR-RFLP, and also by some serological methods such as ELISAs and IFAT [21–23]. Also, other methods such as nested reverse line blot (nRLB) and loop mediated isothermal amplification (LAMP) were also used [24]. Since the disease caused by piroplasms can be difficult to diagnose through direct examination and on the other hand, morphologically, they are very similar to microscopy, so they can be distinguished from each other using serological and molecular methods [23]. In the present study, we used peripheral blood smear and molecular methods for detection of *Theileria* spp. We observed 20 and 30 samples indicated *Theileria* with peripheral blood smear and PCR, respectively. By PCR method all 30 samples were identified as *T. lestoquardi* in sheep and goat in Mazandaran and Golestan provinces. Our study found that PCR method was superior to microscopical examination for detecting *Theileria*, and other studies using different methods found that the ELISA method identified more positive cases. Mazandaran and Golestan are endemic for *Theileria* vectors, and the livestock industry is a common tradition, accounting for roughly half of the industrial in this regions. In some of the world and in different parts of Iran, *T. lestoquardi* is considered as a main causal agent of theileriosis in small ruminants [16,23,25–27]. Study of Heidarpour Bami et al. [19] in the eastern half of Iran using RFLP conducted, out of 132 blood samples, 55.3% were positive for *T. lestoquardi* and 44.7% were positive for *T. ovis* while some studies found *T. ovis* to be more prevalent than *T. lestoquardi*, they also found mixed infection in 3% of cases and explained the main role of *Theileria* spp. vector hosts in the prevalence of theileriosis. In this study, 30 samples were positive for the presence of *Theileria* parasite by molecular method, while by microscopic method, only 20 of them were observed. Also in a similar study conducted by Azizi et al. [28], positive cases were reported by molecular method and microscopic method by 40% and 8.1%, respectively. Furthermore, in a study conducted by Rahmani-Varmale et al. [9], light microscopy of blood smears identified 5.5% of *Theileria* spp., even though nested PCR identified 17% of blood smears as positive. In a similar study conducted by Magzoub et al. [29] in Sudan, the prevalence of *T. lestoquardi* by molecular method was reported to be 13%, which was similar in outcome to the results of this study. The results of the present study on the determination of *Theileria* species with the results of similar studies indicate the high sensitivity of molecular methods compared to microscopic method [28–30].

In conclusion, the results of this preliminary study showed that theileriosis is emerging and enzootic among livestock in the north of Iran and *T. lestoquardi* is the main species of *Theileria* parasite. Also, since the study areas in this study have livestock industry due to climatic characteristics, the results of this study, which for the first time using molecular methods, provided information about the epidemiology of theileriosis in small ruminants in Mazandaran and Golestan provinces. It can be very helpful for health planning and disease control in these areas.

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