First Molecular Evidence of Theileria Lestoquardi in Small Ruminants in Northern Iran

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Research Article

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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 are stained by the Giemsa staining method. As well as for species identification, all samples were examined by molecular method. 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.

Introduction

Ovine theileriosis is an important tick-borne disease of protozoan parasites of the Phylum Apicomplexa named *Thieleria (LiGalon et al. 2020; TumwebazeByamukama et al. 2020).* These parasites are transmitted by ixodid ticks of several genera, including Hyalomma, Amblyomma, Rhipicephalus, and Haemaphysalis(AfshariHabibi et al. 2020; KumarManjunathachar et al. 2020; SpringerShuaib et al. 2020). Theileriosis in wild and domestic animals causes high rates of mortality. This disease is prevalent in the tropical and subtropical areas including East and North of Africa, the Middle East and also some parts of Asia and Europe (GarganoBlanda et al. 2021; Afrasiabian and Yakhchali 2020; DinkelHerndon et al. 2021). Many studies have been presented worldwide on bovine theileriosis but there is a lack of information about ovine theileriosis(Rahmani-VarmaleTavassoli et al. 2019). 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* (*T. lestoquardi*) and the recently described *Theileria spp*. are considered to be extremely pathogenic, while *Theileria ovis* and *Theileria separata* are less pathogenic than *T. lestoquardi*(RazmiYaghfoori et al. 2019). Diarrhea, 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(AginaShaari et al. 2020). Unfortunately, the mechanism of the pathogenesis of malignant ovine theileriosis is not well known yet(Agina et al. 2020). 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 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 (Motevalli HaghiFakhar et al. 2014; MotavalliFakhar et al. 2013). 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 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(Noaman 2014).

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 56º for 2 hours. The mixture was then mixed with phenol chloroform and centrifuged at 13400 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 55º C, and finally kept at -20 degrees until use.

PCR assay

The two genera *Babesia* and *Theileria* were differentiated by PCR and using specific primers derived from hyper variable region of 18srRNA with a piece length of 420-430 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 (ShayanHooshmand et al. 2008).

The PCR reaction with a final volume of 25 microliters, including 12.5 µl master mix, 0.5 µl of each primer, 5µl of DNA template and 6.5 µl deionized water was performed by the Thermo cycler (Bio-Rad).Also the

temperature program used for the PCR reaction: 5 min at 95°C, 38 cycles of 94°C for 45s, 56° for 45s, 72°C for 45s and a final extension step for 10 min. For visual detection by ultraviolet transillumination, we used 1.5% agarose gel electrophoresis with Syber green stain.

Table1. The sequences of specific primers(Shayan et al. 2008)

PCR product (bp) Nucleotide sequences No	
426-430 (*Theileria*) 5 CACAGGGAGGTAGTGACAAG 3	P1
389-402 (*Babesia*) 5 AAGAATTTCACCTATGACAG 3	P2

Detection of *Theileria lestoquardi*

For PCR detection of *T. lestoquardi,* the final volume of the reaction was 25 microliters 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, 45 seconds at 72°C and 45 seconds at 94°C. Finally, PCR was accomplished with the additional extention phase for 10 minutes (Shayan and Rahbari 2007). Finally, 1.5% agarose gel and electrophoresis were used to confirm the amplification

Table2. The sequences specific primers of *T. lestoquardi* (Shayan and Rahbari 2007)

Name	Gene	Nucleotide sequences	PCR product
Theileria Le-sense	*T. lestoquardi* ms1-2 gene AJ006448 NCBI	5·GTTACTCTCACTTCATGTGAG 3·	669 bp
Theileria Le-antisense		5·GGAGAACTTGTCGACAGCTGG 3·	

Results

Twenty (9%) out of 220 livestock were positive by microscopic method (Fig. 1). While a molecular study showed 30 (13.6%) positive for *Theileria* genus (Fig. 2). In two cases, mixed infections with *Theileria* and *Babesia motasi* were also observed. The frequency of *Theileria* infection in Mazandaran and Golestan was 12% (12 of 100) and 15% (18 of 120), respectively. Also, by PCR technique, all positive cases were *Theileria lestoquardi*.

Discussion

Theileria spp. infection is one of the most important protozoal diseases in domestic animals which can cause theileriosis (Shayan and Rahbari 2007). Among the species of *Theileria*, the most virulent and the main cause of fatal disease is *T. lestoquardi*, (BishopMusoke et al. 2004; SivakumarFujita et al. 2019).

Other species such as *Theileria ovis* are less pathogenic and have less economic importance than *T. lestoquardi* (HeydarpourKhazraeinia et al. 2010). 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 (AltayDumanli et al. 2005).

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 (HaghiEtemadifar et al. 2017; SoosaraeiHaghi et al. 2020; SoosaraeiHaghi et al. 2018). Also, in the same study, other methods such as nested reverse line blot (nRLB) and loop mediated isothermal amplification (LAMP) were also used (HassanSkilton et al. 2019). 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. (Soosaraei et al. 2018). 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 and also in two samples showed mixed infection of *Theileria* and *Babesia*. 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 *Babesia* and *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 (Shayan and Rahbari 2007; Soosaraei et al. 2018; GohNgugi et al. 2016; LeemansBrown et al. 1999; Razmi and Yaghfoori 2013). 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 (Rahmani-Varmale et al. 2019). 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, positive cases were reported by molecular method and microscopic method by 40% and 8.1%, respectively. Furthermore, in a study conducted by Rahmani et al., light microscopy of blood smears identified 5.5% of *Theileria* spp., even though nested PCR identified 17% of blood smears as positive (Rahmani-Varmale et al. 2019). In a similar study conducted by Magzoub et al 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(MagzoubEl Ghali et al. 2021). In a study by (Al-HamidhiWeir et al. 2016) respectively. 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(Magzoub et al. 2021).

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.

Declarations

Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval

This work was reviewed and approved by research ethical committee at Mazandaran Mazandaran University of Medical Sciences (IR.MAZUMS.REC.1391.136).

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Figures

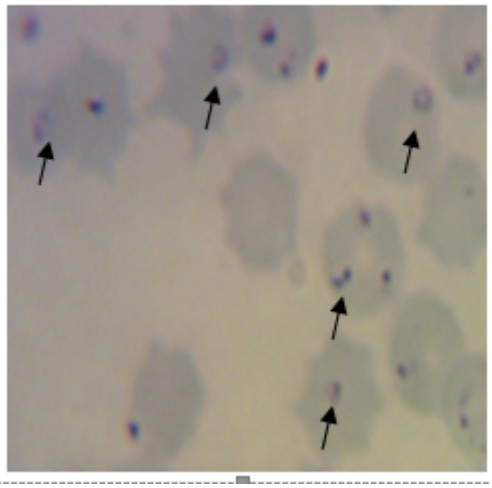

Figure 1

Various forms of Theileria in microscopic examination by Giemsa staining method

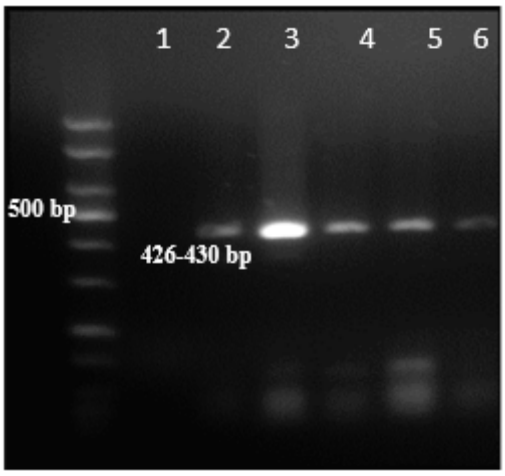

Figure 2

PCR product on gel electrophoresis 1.5%. Line 1 Negative control, Line 2 Positive control, Line, 3, 4, 5 and 6 Theileria samples. M- Marker 100bp