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Short Communication

Molecular and Microscopic Detection of *Theileria* spp. among Cattle and Buffaloes in West Azarbaijan, Iran

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ABSTRACT

Bovine theileriosis is an important tick-borne disease caused by intraerythrocytic parasites from genus *Theileria.* This study sought to detect the theileriosis among cattle and buffaloes using molecular and microscopic tests in West Azerbaijan, Iran. For this purpose, 484 blood samples from 193 cattle and 291 buffaloes were collected during March to July 2014. The breed, gender, age, and habitat of these animals were recorded. These animals were native and apparently healthy, living in four different cities of the province. The blood films were stained with Giemsa's for microscopic examinations. Direct cell semi-nested polymerase chain reaction (PCR) assay was performed to detect *T.annulata* DNA with Tbs-S/Tbs-A and To-S/Tbs-A primer pairs targeted to 18S ribosomal RNA gene for *Theileria* spp. and *T.orientalis* amplification, respectively. The molecular assays revealed that 36 cattle (18.65%) were infected, in which 15 cattle were infected by both *T.annulata* and *T.orientalis*. Out of 291 buffaloes, four samples (1.4%) were infected by *Theileria* genotypes, and two buffaloes (0.7%) were infected only by *T.orientalis.* The observational results of the gender, age, and habitat of the studied animals were similar to animals of the other parts of Iran. The present study indicated that *T.orientalis* may be prevalent in native cattle and buffaloes throughout the northern parts of Iran. This study assessed the infection of buffaloes with *T.orientalis* for the first time.

Keywords: Buffalo, Cattle, Iran, PCR, *Theileria orientalis*

Diagnostic moléculaire et microscopique de *Theileria orientalis* chez les vaches de la province de l'Azerbaïdjan occidental, Iran

Résumé: La theilériose bovine est une maladie importante causée par une cellule intra-érythrocytaire de Theileria. L'objectif de cette étude est de déterminer à l'aide de méthodes moléculaires et microscopiques l'état actuel de l'infection de Theileria chez les vaches de la province de l'Azerbaïdjan occidental en Iran. Dans cette étude, 484 échantillons de sang ont été prélevés entre mars et juillet 2014 à partir de vaches et buffles indigènes provenant de quatre villes différentes. Les animaux étudiés avaient été classés selon la race, le sexe, l'âge et la ville. Tous les échantillons sanguins des animaux étudués ont fait l'objet d'une coloration de Giemsa pour les observations parasitologiques . Les techniques de PCR et semi-nested PCR ont été également utilisées pour détecter l'ADN de *Theileria orientalis* à l'aide des paires d'ARN ribosomiques 18S: les ensemble d'amorces Tbs-S/Tbs-A et To/Tbs-A ont été respectivement utilisés pour l'amplification de *Theileiria*, et *Theileria orientalis*. . Les analyses moléculaires sur 484 échantillons ont détecté 40 échantillons infectés par le Theileria (8,26%) et 17 (3,51%) échantillons positifs pour le *Theileria orientalis*. La prévalence de *Theileria orientalis* chez les vaches indigènes et les buffles était respectivement de 8.33% et 0.68%, révélant pour la première fois une différence significative entre ces deux groupes ($p<0.05$). L'utilisation de cette approche moléculaire a

confirmé l'existence d'infection au *Theileria orientalis* chez les vaches indigènes ainsi que chez certains buffles originaires plus particulièrement du nord-ouest de l'Iran.
Mots-clés: *Theileria orientalis*, buffles, vaches, réaction en chaîne de polymérase, Iran

INTRODUCTION

Bovine theileriosis caused by infection with an apicomplexan parasite, *Theileria spp.*, is a tick-borne disease which annually leads to a major economic loss in industrial livestock at endemic sites. This disease is manifested by fever, anemia, dyspnea, icterus, hepatosplenomegaly, gastroentritis, lymphadenopathy, and even death (Uilenberg, 1981). The milk production will be adversely affected in case of subclinical infection (Savini et al., 1998). The bovine and ovine theileriosis caused by *T.annulata* was reported from some parts of Iran (Hashemi-Fesharki et al., 1998; Safarpour Dehkordi et al., 2012). To the best of our knowledge, except the Akbari et al. (2012) study which is a preliminary molecular survey on *T.annulata* among cattle of West and East Azarbaijan provinces of Iran using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) method, no comprehensive survey has been performed in West Azarbaijan particularly on the cattle and buffaloes. This was the main motivation for conducting this study on theileriosis of cattle and buffaloes in West Azarbaijan.

Differentiation of the *Theileria* species based on the morphological data, host specificity, and route of transmission by ticks is recently understood. Sometimes a light microscope could not distinguish the *Theileria* spp. from some *Babesia* spp. in Giemsa-stained blood smears (Ahmed et al., 2006). Therefore, to detect the *Theileria* spp. in apparently healthy cattle and buffaloes, we need to us semi-nested PCR assay and compare the results with the findings of microscopic examinations of blood smear.

MATERIALS AND METHODS

Sampling. West Azarbaijan (37.54° N, 45.07° E) is in North-West portion of Iran. The studied area is a mountainous region with a cool climate (Figure 1). This province is adjacent to Turkey with 433 Km border. The sample size was calculated based on the expected prevalence of 5% and desired absolute precision of 95%. A total of 484 blood samples were collected randomly from 193 native cattle and 291 buffaloes during March to July 2014. In this study, the semi-nested PCR technique was used to detect the *Theileria* spp. in the apparently healthy cattle and buffaloes. The blood sampling was performed by taking 5 ml of blood from each animal. The samples were kept in sterile Venoject glass tubes and fixed by same volume of 70% concentration of ethanol. Additionally, two blood smears were prepared. The blood smears were air-dried and fixed by methanol (99%) for five minutes.

Microscopic examination. The blood films were stained with Giemsa's according to a method described by kelly in 1979. Briefly, the fixed smears were stained with 5% Giemsa diluted in phosphate buffer (pH 7.2). The analysis was performed by microscopic examination.

DNA Extraction. DNA extraction performed using molecular biological system transfer kit (MBST, Iran), according to the manufacturer's instructions. The technique based on the selective binding of the nucleic acids to a silica-based membrane which was placed in the MBST column. Briefly, a little amount of fixed blood sample was placed in a 1.5 ml microcentrifuge tube and completely dried overnight at room temperature. After that, the samples were lysed in 180 µL of lysis buffer and proteinase K at 55 °C until the samples were completely dissolved. After addition of 360 µL binding buffer and incubation for 10 min at 70ºC, 270 µL of ethanol (100% Merck) was added to the solution. Then, it was vortexed and the complete

solution was transferred to the MBST column. The columns were washed twice with washing-buffer at 8000×g. The DNA was eluted from the carrier using elution buffer and stored at -20 °C; and it was visualized on 1.5% agarose gel (Shayan et al., 2016).

PCR and nested-PCR. Bovine beta-actin gene was amplified using the primer pairs of P1 (β-actin sense) and P2 (β-actin antisense) to confirm the suitability of extracted DNA for PCR amplification (Table 1). Approximately, 10 ng DNA solution was used for the PCR which performed in a total reaction volume of 100 µl containing 10X reaction buffer, 2.5 unit of Taq DNA polymerase (Cinnagen, Iran), 2 µl of each primer (20 µM, Cinnagen, Iran), 2 µl of each dATP, dTTP, dCTP, and dGTP (200 µM, Fermentas), and 1.5 mM $MgCl_2$.

The amplification was executed in an automated thermocycler (MWG Biotech Inc Primus, Germany) under following program: initial denaturation at 95 °C for 5 min followed by 34 cycles at 94 °C for 45 sec (denaturating step), 60 °C for 45 sec (annealing step), and 72 °C for 45 sec (extension step) with an additional extension step at 72 °C for 5 min. The expected PCR product size for β-actin was 686 bp. The 18S ribosomal RNA (rRNA) gene PCR amplification was used to detect the infection of *Theileria spp.* and *Babesia spp*. among the samples. Primer pairs were designed based on the nucleotide sequences of 18S rRNA gene of *Theileria* and *Babesia*. The P3 primer sense was derived from 426-430 bp in *T.annulata* (accession no. AY150056.3). The P4 primer antisense was derived from 639 bp of the same gene. The PCR of β-actin gene was performed with the same program at the distinct annealing temperature of 54 °C. The expected size of PCR products for *Theilera spp*. and *Babesia spp*. were 426-430 bp and 389-402 bp, respectively (Shayan et al., 2016). The semi-nested PCR procedure was used to differentiate between *T. annulata* and *T. orientalis*. For this purpose, two forward primers of P5 and P6 with the PCR product size of 193 bp and 235 bp, which are specific for *T.annulata* and *T.orientalis*, respectively, and a P4 reverse primer were used. The annealing temperature for both species was 54 °C (Table 1).

Table1. The Primers used for identification of Bovine *T.annulata* and *T.orientalis*

No	Name of primers	Gene	Nucleotide sequences
P1	Bba-S	Bovine β - Actin	5` CCT-AGA-GAG-AAG-CGG-GGT-G-<G> 3`
P2	Bba-A	Bovine β - Actin	5` ATC-ACT-GCC-CTG-GCA-CCC-A-<G> 3`
P3	Tbs-S	18S rRNA*	5` CAC-AGG-GAG-GTA-GTG-ACA-AG 3`
P4	Tbs-A	18S rRNA	5` CTA-AGA-ATT-TCA-CCT-CTG-ACA-G 3`
P5	Ta-S (specific for *T. annulata*)	18SrRNA	5` ACG-GAG-TTT-CTT-TGT-CTG-<A> 3`
P6	To-S (specific for *T. orientalis*)	18SrRNA	5` ACA-TTT-CTC-TTG-TTT-GAG-<T> 3`

*** Ribosomal RNA**

Statistical Analysis. Data analysis was carried out using SPSS version 22. In all measurements P-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Microscopic examinations. Regarding the obtained results, 25 (13%) of cattle and four (1.4%) of buffalo samples exhibited *T.annulata* piroplasms. The morphological features of *T.orientalis* are undetectable by using a light microscope; therefore, in this study, *T.orientalis* was not found among the smears (Table 2).

Semi-nested PCR findings. Thirty six (18.65%) cattle samples and four (1.4%) buffalo samples were positive for *T.annulata*. Mixed infection with both *T.annulata* and *T.orientalis* was found in 15 (7.8%) cattle samples. Similarly, out of four buffalo samples, two (0.7%) smears were infected with *T.orientalis* or both piroplasms (Table 2). As demonstrated in Table 2, a significant difference was observed between cattle and buffaloes in terms of infection with *T.annulata* ($P<0.05$; Table 2). According to the results of the current study, the prevalence of theileriosis (the infection of *T.annulata)* through West Azarbaijan was similar to the other regions of Iran (Noaman, 2013;

Table 2. Detection of *Theileria* spp. among cattle and buffaloes of West Azarbayjan

Theileria spp.	Cattle					Buffaloes				
	No. Examined	Microscopic Examination		Molecular assays*		No. examined	Microscopic Examination		Mol Molecular assays*	
		No. infected	%	No. infected	%		No. infected	%	No. infected	%
T.annulata	193	25	**13**	36	**18.65**	291	4	**1.4**	4	**1.4**
T.orientalis	193	0	**0**	15	**7.8**	291	0	**0%**	2	**0.7**

*Semi-nested PCR

Hoghooghi-Rad et al., 2011; Hashemi-Fesharaki, 1988). The bovine theileriosis has been reported in Iran from 70 years ago (Hashemi-Fesharki, 1988). The early diagnosis of theileriosis was based on microscopic examinations. More recently, some serological tests such as indirect fluorescent antibody (IFAT) and enzyme-linked immunosorbent assay (ELISA) tests and finally molecular assays were applied for specific diagnosis of theileriosis (Shayan and Rahbari, 2005; Shayan et al., 2016). The prevalence of the bovine theileriosis in different parts of Iran varied from 3.4% to 18.5% and recently it was reported as 23.9% (Noaman, 2013). The present study revealed that *T.annulata* was the most prevalent apicomplexan protozoa among the cattle (18.65%) and buffaloes (1.4%) in West Azarbaijan, Iran (Table 2). Iran) may strengthen the hypothesis about the vast distribution of this protozoa through the northern parts of this country (Hoghooghi-Rad et al., 2011). According to Hayashida et al. (2012), *T.orientalis* was assigned to four genotypes of Ikeda or type 1, Chitose or type 2, Buffeli or type 3, and 4. However, other studies reported that there are five types of this parasite, classified by their major piroplsm surface protein (MPSP) and p23 (Ota et al., 2009; Yokoyama et al., 2011). The distribution of Ikada type is limited to Eastern Asia entailing Japan, South Korea, North of China, and Australia. The livestock could easily The interesting matter of the present survey was on the presence of *T.orientalis/buffali/sergenti* complex among these mammalians in West Azarbaijan, Iran (Altay et al., 2008; Yokoyama et al., 2011). The high prevalence of *T.orientalis* in Golestan (North-East of succumb due to severe clinical signs of the theileriosis (Hayashida et al., 2012). Regarding the literature, it was confirmed that *T.orientalis* is nonpathogenic and not responsible for economic loss in domestic livestock, while to the best of our knowledge, there is no study about the diverse types of *T.orientalis* (Uilenberg and Hashemi-Fesharki, 1984; Ghaemi et al., 2012). Therefore, such a deduction is going to be accepted unless more attempts be done on identification of the types of this protozoa. In West Azarbaijan, 18.65% of the cattle were infected by *T.annulata* and 7.8% of them revealed a mixed infection with *T.orientalis* and *T.annulata*. Nevertheless, 1.4% of the buffaloes were infected by *T. annulata* and 0.7% of them were inflicted with the *T.orientalis* (Table 2). The studied cattle and buffaloes were not clinically infected; therefore, they were suitable reservoirs for *Theileria* spp. for the other ruminants. Regarding a long adjacent border between West Azarbaijan of Iran and Turkey, it is worth to evaluate the risk of transfer of the disease through this border (Figure 1). The animal farmers are occasionally engaged in the long-haul transport of livestock such as cattle along this border. As, several evidences indicated that the *T.orientalis* is quite prevalent among cattle in eastern parts of Turkey (Dumanli et al., 2005; Aktas et al., 2006; Altay et al., 2008). Rahbari et al. (2007) determined the *Hyalomma anatolicum excavatum, Boophilus annulatus*, and *Rhipicephalus bursa* as the main vector ticks for *Theileria*.spp in eastern Turkey, which are present in Iran as well. The transportation of cattle from Turkey may cause the dissemination of

Theileria spp. in Iran. This hypothesis needs to be tested by diagnostic procedures in imported animals.

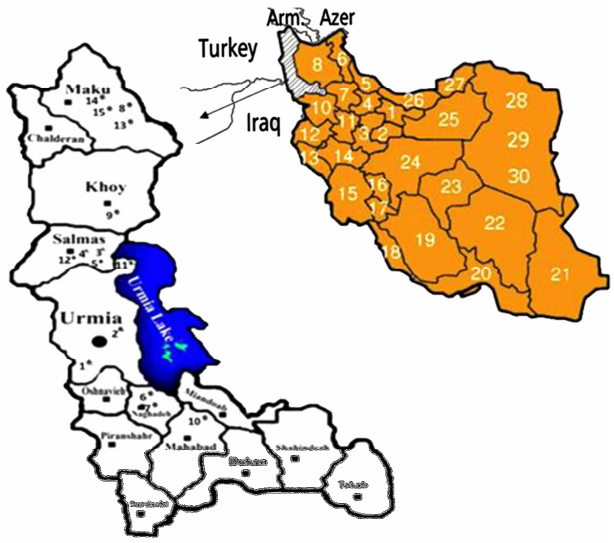

Figure 1. The map of western Azarbaijan province which is the 9th province of Iran.

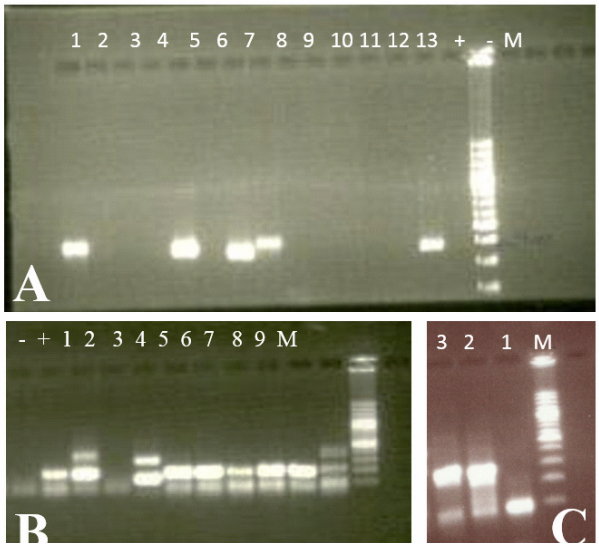

Figure 2. A: DNA was extracted from the blood samples and amplified with primers P1 and P2 to recognize the *Theileria spp*. infected blood samples. B: The PCR product achieved with primers P3/P4 were amplified using primers P5/P4 to recognize the *T. annulata* infected samples. C: The PCR product achieved with primers P3/P4 were amplified using primers P6/P4 to recognize the *T. orientalis*.

Discrimination between piroplasms of different *Theileria* species based on the morphometric criteria and staining methods would not give rise to satisfactory results. The semi-nested PCR indicated that 18.65% of the cattle were infected with *T.annulata*, while this rate was 13% by microscopic examination(Figure 2)(Table 2). These intraerythrocytic piroplasms are highly pleomorphic; therefore, the distinction between *T.annulata* and *T.orientalis* using a light microscope is not reliable (Almeria et al., 2001; Ota et al., 2009; Yokoyama et al., 2011; Shayan et al., 2016). However, the presence of rod- and veil-like structures in the cytoplasm of the infected erythrocyte, under an electron microscope, is diagnostic for *T.orientalis* piroplasm (Almeria et al., 2001; Aktas et al., 2006). The theileriosis did not lead to economic loss in West Azarbaijan up to now. The wide administration of acaricides such as flumethrin and cypermethrin did not eliminate the tick population (Rahbari et al., 2007). Although, these agents might be effective in some area, perhaps the disadvantages of such chemicals induce enormous economic loss due to tick resistance, impacts on feeding and health status of the animals, and changing the ecosystem (Mehlhorn, 2008). So far, it was believed that using efficient acaricides, changing the habitats of tick, treatment of infected animals, and selection of resistant animals may lead to elimination of the population of the ticks. After years of endeavors, no significant limitation of theileriosis was observed by using these methods (Rahbari et al., 2007). It is recommended to protect the farm animals against ticks by producing recombinant antigens as it was followed up in some countries against *Boophilus microplus,* by manufacturing Bm86 and Bm91 antigens and using them as vaccines to protect the domestic animals (Willadsen et al., 1996; García-García et al., 2000; Rodriguez-Valle et al., 2012). Theileriosis is prevalent among cattle and to a lesser extent in buffaloes in West Azarbaijan. The *T.annulata* is the main causative agent of theileriosis and sometimes it led to a mixed infection with *T.orientalis*. Previous studies did not report this infection among the buffaloes. Probably, *T.orientalis* is imported from a neighboring country. As a result, vaccination of the livestock, with recombinant antigens

prepared from tick vectors, is recommended to eliminate the risk of tick-borne diseases.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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