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Detection of Native Carrier Cattle Infected with *Theileria annulata* by Semi-Nested PCR and Smear Method in Golestan Province of Iran

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Abstract: *Theileria annulata*, as a pathogenic agent of tropical theileriosis, gives rise annually to serious economic losses in cattle industry of Iran. The carrier cattle, harbouring the latent forms of *Theileria annulata,* play a major role in infecting tick vectors and in disseminating the infection. The aim of this study was determining the carrier cattle, infected with *Theileria annulata* in Golestan province of Iran by semi-nested PCR method in comparison with Smear method. In this study 160 blood samples, collected from apparently healthy native cattle, were examined. The Tbs-S/Tbs-A primer set was used for PCR amplification of *Theileria* sp. and the Ta-S/Tbs-A specific primer set was used in semi-nested PCR technique for detection of *Theileria annulata*. Blood smears of each case were examined by Giemsa staining method, too. The semi-nested PCR accurately revealed 12 (7.5%) positive samples; whereas Giemsa staining method could detect 6 (3.75%) out of 160 blood samples. The results indicated that Giemsa staining method, having 50% sensitivity, is not suitable for determination of *Theileria annulata* carrier cattle, while semi-nested PCR technique can be used as a Gold standard method for the mentioned aim and also can be used for screening of *Theileria annulata* carrier cattle in all regions.

Key words: Theileriosis · Cattle · Northeast of Iran

INTRODUCTION

Mediterranean theileriosis caused by *Theileria annulata* is distributed in North Africa, Southern Europe, Middle East and India. This disease causes high mortality and morbidity rates thereby resulting huge economic losses to the cattle industry in Iran too [1-4]. Ticks of the Genus *Hyalomma* are the common vectors of this protozoan parasite [5-7]. The cattle of different parts of Iran are affected by *Theileria annulata*. The native breeds are more resistant to theileriosis and are affected by subclinical form of the disease whereas non-native cows are very sensitive to this infection and if they are not treated effectively; their mortality rate will rise up to 40-60% and even more [8, 9].

Usually, this intracellular protozoa remains covert in the cattle after complete recovery from acute phase of the disease. Sometimes, premunition occurs with very few numbers of the parasite hidden in the body which renders the animal a partial protection towards the invasion of new sporozoites introduced by an infected tick. Moreover, vaccination of cattle against theileriosis, using attenuated cultured schizonts supplies the animal to carry a few parasites [10]. All these cited events render the cattle to be a potential carrier in the area. Thus, the native carrier cattle are the major agents of spreading the infection among the ticks and the herds of cattle [11]. In the carrier cattle, the numbers of piroplasms are very scarce; therefore, finding the parasite is often tiresome [9].

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In Iran, the Giemsa staining of the blood smears is the common method for the identification and characterization of the piroplasm. This method is not sensitive [12] and confronts some false positive or false negative results. Meanwhile, serological procedures are usually employed in determining subclinical infections. False positive and false negative results are also commonly observed in serological tests due to cross-reactions or sometimes inadequate antibody level in carriers due to the long term infection [13].

The PCR method is more accurate in comparison with the serological tests such as enzyme linked immunosorbent assays (ELISA), immunofluorescent antibody test (IFAT) and indirect haemagglutination assay (IHA) as well as microscopic detection of piroplasmic forms. Using PCR method enables us to detect parasitic infections with clinical or without clinical signs [5,14, 15].

The present study was aimed to report the number of native carrier cattle infected with *Theileria annulata* in Golestan province of Iran and the usage of the semi-nested PCR method in diagnosing actual carriers in comparison with the traditional Giemsa staining method.

MATERIALS AND METHODS

Study Area and Collection of Samples: The survey was conducted during 2009 to 2010 in Golestan Province of Iran. A total number of 160 blood samples were collected from apparently healthy native cattle. From each cattle a thin layer blood smear was prepared from its ear vein. Also 5 ml whole blood sample was taken from caudal vein of each cattle. The age of the cattle were more than 1 year and all of them were females and reared under open system management.

Microscopic Examination: Blood smears were prepared according to Kelly (1979). After cleaning the ear with 70% ethanol, a small drop of ear blood was stocked on a clean slide glass. The small blood drop was rapidly spread into an even thin film by a second clean slide held at 45°angle and immediately dried. The slide was labeled and kept in upright position in a special box and was carried to the laboratory. The blood smears were quickly fixed by methanol (99%) for 5 min and stained in Giemsa stain diluted at 5% with buffer solution for 30 min and flushed with tap water, then left to dry. Subsequently, the stained blood smears were examined with an oil immersion lens at a total magnification of x1,000 for the presence of *Theileria* piroplasms. Each blood smear was examined twice before being considered negative [16].

DNA Extraction from Whole Blood Samples: DNA was extracted from whole blood samples using DNA extraction Kit (MBST, Iran) according to the manufacturer's instructions. This method is based on the specific binding of the DNA to the carrier. Briefly, a piece of fixed blood was first lysed in 180 µl lysis buffer and the proteins were degraded with 20 µl proteinase K for 10 min at 55°C. After addition of 360 µl binding buffer and incubation for 10 min at 70°C, 270 µl ethanol (100%) was added to the solution and after vortexing, the complete volume was transferred into the MBST-column. The MBST-column was first centrifuged, then washed twice with 500 µl wash buffer. Finally, DNA was eluted from the carrier in 100 µl elution buffer. 10µl of extracted DNA was analyzed on a 1% agarose gel in a TBE buffer at 100 V for 45 min and then visualized under UV light after staining with ethidium bromide.

Polymerase Chain Reaction (PCR): Approximately 10 ng DNA solution was used for the PCR analysis. The PCR was performed in 100 µl total volume including 10x PCR buffer, 2.5 U Taq Polymerase (Cinnagen, Iran), 20 µM of each primer (Cinnagen, Iran) (Tab.1), 200 µM of each dATP, dTTP, dCTP and dGTP, (Fermentas), 1.5mM MgCl2, in automated Thermocycler (MWG Biotech Primus, Germany) with the following program: 5 min incubation at 95 C to denature double strand DNA, Two cycle of 94°C for 45 s (denaturing step), 55 or 60°C for 90 s (annealing step) and 45 s in 72°C (extension step) that followed by two cycles of 94°C for 45 s, 55 or 60°C for 60 s and 45 s at 72°C. This step was followed by 34 cycles of 45 s at 94°C, 45 s at 55 or 60°C and 45 s at 72°C. Finally, PCR was completed with the additional extension step at 72°C for 5 min. Annealing temperature used for Bba-S/Bba-A primer set was 60°C and for the Tbs-S/Tbs-A primer set was 55°C. All primers were designed in Investigating Unit of Molecular Biological System Transfer (MBST, Iran) and were synthesized by Cinnagen Company. The primers used in this study are listed in the Table 1. The Bba-S/Bba-A primer set was used for PCR amplification of Bovine Beta-Actin encoding gene and the amplified sequence weight by this primer set was 639 bp.

Table 1: Primers used for the amplification of the DNA

Primer name	Gene	Nucleotide sequences (5' -3')	Specificity
Bba-S	Bovine Beta-Actin	CCT-AGA-GAG-AAG-CGG-GGT-G-<G>	For PCR amplification of Bovine β-Actin encoding gene
Bba-A	Bovine Beta-Actin	ATC-ACT-GCC-CTG-GCA-CCC-A-<G>	For PCR amplification of Bovine β-Actin encoding gene
Tbs-S	18S rRNA	CAC-AGG-GAG-GTA-GTG-ACA-AG	Specific for *Theileria sp.* and *Babesia sp.*
Tbs-A	18S rRNA	CTA-AGA-ATT-TCA-CCT-CTG-ACA-G	Specific for *Theileria sp.* and *Babesia sp.*
Ta-S	18S rRNA	ACG-GAG-TTT-CTT-TGT-CTG-<A>	Specific for *Theileria anuulata*

Also the Tbs-S/Tbs-A primer set was used for PCR amplification of 18SrRNA of *Theileria* sp. and *Babesia* sp. and the amplified sequence weight by this primer set for *Theileria* sp. and *Babesia* sp. were 426-430 and 389-402 bp, respectively. Distilled water was used as negative control in each PCR reaction.The DNA from *Theileria annulata* infected cattle obtained from department of parasitology, Faculty of veterinary medicine, University of Tehran, Iran, was used as positive control for corresponding primers. For analysis the PCR product, 10µl of each of them were subjected to electrophoresis on a 2% agarose gel in a TBE buffer at 100 V for 45 min and then visualized under UV light after staining with ethidium bromide.

PCR Product Purification: PCR products were purified using rapid PCR product purification Kit (MBST, Iran) following the protocol of the manufacturer**.** Briefly, 200 µl of binding buffer added to 100 µl PCR sample and mixed by vortexing. After addition of 150 µl ethanol (100%) to the solution and after vortexing, the complete volume was transferred to the MBST-column. The MBST-column was first centrifuged, then washed twice with 500 µl wash buffer. Finally, DNA was eluted from the carrier in 100 µl elution buffer and used for semi-nested PCR.

Semi-Nested PCR: In order to show that the first PCR product (using Ts-S/Ts-A primer set) was *Theileria annulata,* it was first purified and amplified using Ta-S/Tbs-A primer set derived from the 18SrRNA encoding gene. The amplified sequence weight by this specific primer set was 193 bp. Briefly, Approximately 10 ng DNA solution from purified PCR product was used for the semi-nested PCR analysis. The semi-nested PCR was performed in 100 µl total volume including 10x PCR buffer, 2.5 U Taq Polymerase (Cinnagen, Iran), 20 µM of each primer (Cinnagen, Iran) (Tab.1), 200 µM of each dATP, dTTP, dCTP and dGTP, (Fermentas), 1.5 mM MgCl2, in automated Thermocycler (MWG Biotech Primus, Germany) with the following program: 5 min incubation at 95 C to denature double strand DNA, Two cycle of denaturing double strand DNA in 94°C for 45 s (denaturing step), annealing in 55°C for 90 s (annealing step) and 45 s in 72°C for extension (extension step), followed by 35 cycles of 45 s at 94°C, 45 s at 55°C and 45 s at 72°C. Finally, semi-nested PCR was completed with the additional extension step for 5 min in 72°C. For analysis the semi-nested PCR product, 10µl of each of them were subjected to electrophoresis on a 2% agarose gel in a TBE buffer at 100 V for 45 min and then visualized under UV light after staining with ethidium bromide.

RESULTS

In this study all the extracted DNA samples analyzed by 1% agarose gel electrophoresis and showed all of them were positive (Fig. 2). Also all the extracted DNA samples amplified using the Bba-S/Bba-A primer set and analysis of these PCR products on 2% agarose gel electrophoresis showed all of them were positive (Fig.2).

After the amplification the DNA sample using Tbs-S/Tbs-A primer set for PCR and analysis these PCR product on the 2% agarose gel electrophoresis, 13 out of 160 blood samples (8.12%) were positive for *Theileria* sp. and none of them was positive for *Babesia* sp. (Fig.2).

Having purified PCR product positive samples, all of them were then amplified by semi-nested PCR with *Theileria annulata* specific primers. In semi-nested PCR, 12 out of 13 blood samples were positive for *Theileria annulata* but only 1 sample was negative. So the rate of *Theileria annulata* carrier cattle in Golestan province by semi-nested PCR method was 7.5% (12 out of 160) (Table 2).

Result of this step compared with Giemsa staining method for each sample. By microscopic examination 10 out of 160 blood smears (6.25%) was positive for piroplasmic forms of *Theileria annulata*. But 4 out of 10 (40%) positive samples of Giemsa staining method were

Table 2: Comparison between semi-nested PCR and Giemsa staining method in detection of *Theileria annulata* in native carrier cattle of Golestan province

		Giemsa staining method		
Golestan Province		Positive	Negative	Total
Semi-Nested PCR method	Positive	6	6	12
	Negative	4	144	148
	Total	10	150	160

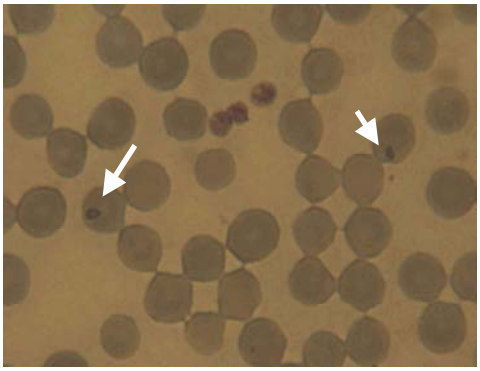

Fig. 1: Piroplasmic forms of *Theileria annulata* in a microscopic field by Giemsa staining method

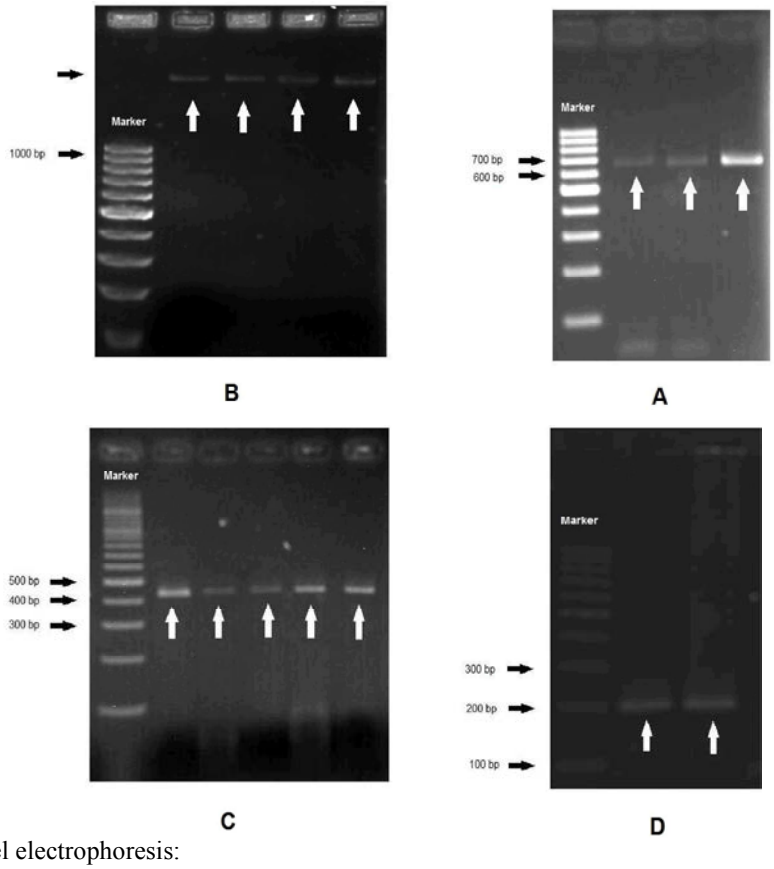

Fig. 2: Agarose gel electrophoresis:
A- Analysis of genomic DNA extracted from whole blood samples of cattle that fixed in ethanol
B- Analysis of PCR amplification using Bba-S/Bba-A primer set derived from the Bovine Beta-actin encoding gene
C- Analysis of PCR amplification for detection of *Theileria sp.* by using Tbs-S/Tbs-A primer set derived from the 18SrRNA encoding gene
D- Analysis of semi-nested PCR amplification for detection of *Theileria annulata* by using Ta-S/Tbs-A primer

set derived from the 18SrRNA encoding gene

negative by semi-nested PCR (False positive). Also 6 out of 12 (50%) were positive by semi-nested PCR but negative with Giemsa staining method (False negative) (Table 2). If false positive cases are subtracted from results of Giemsa staining method, thus, the actual rate of native carrier cattle infected with *Theileria annulata* by this method will be 3.75% (6 out of 160).

DISCUSSION

Tropical theileriosis is a lymphoproliferative and intraerythrocytic disease, occurring within two phases, in infected cattle. It is considered as one of the important diseases which produce large economic losses in every involved country. Carrier cattle are the main source of persistence of theileriosis in any area where the appropriate tick vectors are existing. The cattle become carriers when they pass the acute phase of the disease. After recovery, low numbers of *Theileria annulata* piroplasms still remain in the body. Meanwhile, vaccinated cattle harbour this intraerythrocytic piroplasm in a latent form. The carrier cattle are apparently healthy with no sign of the contraction. However, they are really very important in terms of delivering the infection to ticks. The climatic situations of tropical and subtropical regions in Iran prepare optimum conditions to distribution of *Hyalomma* ticks as the main vector for tropical theileriosis in the most parts of the country. Thus, a precise recognition of carrier cattle is desired for plotting control plans for the restriction of the disease dispersion, not to be extended to other areas, at least. Also, early diagnosis of theileriosis requires a simple and precise method with the capability to recognize infecting agents in very low density in animals.

In this study two main diagnostic methods including Giemsa staining, as the common procedure in Iran and semi-nested PCR, as the highly sensitive and specific method, were used to detect the rate of native carrier cattle harboring *Theileria annulata* in Golestan province in northern part of Iran.

In order to approve the presence of genomic DNA in extracted DNA samples, all of the extracted DNA, analyzed by 1% agarose gel electrophoresis, proved to be positive (Fig.2). Also for control the ability of genomic DNA in extracted DNA samples for PCR, all the extracted DNA amplified, using the Bba-S/Bba-A primer set and analysis of these PCR products on 2% agarose gel electrophoresis, were shown to be all positive too (Fig.2).

For preventing any misdiagnosis of mixed infections of *Babesia* and *Theileria* piroplasmic organisms in erythrocytes, the common primer set (Tbs-S/Tbs-A), derived from 18srRNA encoding gene, was designed and used in this study to amplify the both Genus of *Theileria* and *Babesia* species in PCR step. Electrophoresis on agarose gel could distinguish these two PCR products, indicating no *Babesia* infection was detected in the present study.

After purification of PCR product positive samples, all of them were amplified by semi-nested PCR with *Theileria annulata* specific primer set (Ta-S/Tbs-A) derived from 18srRNA encoding gene. In semi-nested PCR, 12 out of 13 blood samples were positive for *Theileria annulata* but only 1 sample was negative. So the results of semi-nested PCR showed that most of the carrier cattle detected in PCR step, infected with *Theileria annulata*. Also the result of this study suggested that other *Theileria* species besides *Theileria annulata* are also present in Golestan province of Iran, because one sample gave positive result by PCR for *Theileria* sp. but was not amplified in semi-nested PCR with *Theileria annulata* specific primer. This could be non-pathogenic *Theileria* species such as *Theileria sergenti*, *Theileria buffeli* or *Theileria orientalis*.

In microscopic examinations of 160 cattle blood smears, we could only detect 10 (6.25%) cases containing the *Theileria* piroplasms. In order to ascertain these 10 cases are really *Theileria annulata*, we examined them with semi-nested PCR which revealed only 6 cases were positive for theileriosis. In other words, the false positive rate of the Giemsa staining method is 40%. If we subtract 4 from 10 cases then the true positive rate of Giemsa staining method will be 6 out of 160 samples or 3.75%. Also, we carefully examined the blood smears of 12 semi-nested PCR positive cases. We could only find the *Theileria* piroplasms in 6 smears which is indicative of 50% sensitivity of Giemsa staining procedure.

Several studies have documented the PCR assays are more sensitive and specific than conventional diagnostic techniques in determining carrier animals. For example, D`oliveira *et al*. [5] examined 92 blood samples collected from cattle in Spain and tried to diagnose *Theileria annulata* by PCR, IFA and Smear methods. The positivity rates of the three procedures were 75%, 40% and 22% respectively. Also, Roy *et al*. [17] reported that the

number of positive cases of 50 blood samples of native cattle by PCR and Smear method were 22 and 8, respectively. Ameen *et al*. [18] could only detect 4.1% trypanosomosis among the local ruminants in Nigeria. El-metanawey *et al*. [19] compared the results of PCR which revealed 93.8% of experimentally infected goats by *Trypanosoma evansi* with those of Giemsa staining method which only could reveal 13.8% of the experimentally goats.

In Iran, Azizi *et al* [20] reported the positive infection rate of 140 carrier cattle was 40% by PCR and only 8.1% by Giemsa staining method. Youssefi *et al*. [21] examined 103 blood samples of pigeons from Golestan province only by Giemsa staining method. They reported that only 17.4% harboued *Haemoproteus columbae*. On the other han, Mahmmod *et al*. [22] examined 30 blood samples of cattle and water Buffalos by PCR and Giemsa staining method in Egypt. They showed that the prevalence of *Theileria* infection by PCR assay was 70% (21 out of 30) while by Giemsa staining method was 30% (9 out of 30). Bahadori *et al*. [23] reported that the infection rate of theileriosis in native cattle of Golestan province was 7.33% by Giemsa staining method. This figure is similar to our finding which showed 6.25% (10 positive cases out of 160 samples) by the same method. According to the above explanations, these figures may not be reliable, because the sensitivity of this method may not be more than 50%.

Semi-nested PCR designed in this study based on detecting *Theileria annulata,* was capable of showing the cases which their Giemsa stained blood smears were false negative or false positive in visual examination under light microscope. It seems that the visual mistakes of the examiner may be due to very low parasitaemia, 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.

In conclusion, the PCR and especially semi-nested PCR are highly specific and sensitive methods for identifying the species of *Theileria annulata* and screening the carrier cattle in the epidemiological surveys. On the other hand, Giemsa staining method is not suitable for detecting the carrier or chronic phases of theileriosis, although it is an easy and fast diagnostic technique for detecting this infection in acute phase with clinical signs in cattle.

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