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ORIGINAL ARTICLE

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Comparison of molecular and microscopic technique for detection of *Theileria* spp. in carrier cattle

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Abstract In Iran, theileriosis is normally diagnosed with traditional Giemsa staining method. This is not applicable for identification of the carrier animals. The aim of this study was to compare conventional Giemsa staining method with the PCR technique in the detection of *Theileria* organisms. In this study, examinations were performed on 150 blood samples from cattle without clinical signs. Sensitivity and specificity of 50 microscopic fields were compared with *Theileria* specific PCR. The degree of agreement between PCR and microscopic test was determined by Kappa (κ) values with 95 % confidence intervals. PCR showed that 42 samples were *Theileria* spp. positive, while routine microscopy showed erythrocytes harboring *Theileria* like structures in 11 blood samples. Examination of 50 microscopic fields showed 57 % sensitivity and 99 % specificity compared to 100 % sensitivity and specificity for PCR. The κ coefficient between PCR and Microscopy (50 fields) techniques indicated no level of agreement. Our results showed that the microscopic examination remains the convenient technique for day-to-day diagnosis of clinical cases in the laboratory but for the detection of carrier animal containing low parasitemia. Therefore, molecular methods such as PCR can be used as a safe method for identifying cattle persistently infected with *Theileria* spp.

Keywords *Theileria* · Carrier cattle · Microscopic method · Giemsa staining · PCR

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Introduction

Theileriosis is the main tick-borne diseases in tropical and sub tropical regions (Uilenberg 1981). Tropical theileriosis caused by *Theileria annulata* is an endemic and economically important disease of cattle especially in pure Holsteins and crossbreds (Hashemi-Fesharki 1988). Hyalomma, vectors of causative organism of tropical theileriosis, are most abundant ticks recorded throughout Iran (Rahbari et al. 2007). The disease is characterized by a marked rise in body temperature, reaching 40–41.5 °C, depression, lacrimation, nasal discharge, and swelling of the superficial lymph nodes and anemia (Gubbels et al. 2000). The recovered cows from acute or primary theileriosis, remain infected for a long period and even for the rest of their lives, so acting as reservoirs of infection for ticks and cause natural transmission of the disease (Cacci et al. 2000; Kirvar et al. 2000). Therefore, detection of *Theileria* in asymptomatic carrier cattle is important for the implementation of successful control programmes.

Conventional method for identification includes examination of blood smears using Giemsa staining, which is accompanied with some critical problems. Giemsa-stained blood smears can be indeed used as a suitable method to detect *Theileria* agents in the animals clinically suspected acute theileriosis, but it is not applicable for the determination of pre-symptomatic or carrier animals, where parasitemia is very low (Friedhoff and Bose 1994).

Several serological tests have been established for detection of subclinical infections in epidemiological studies. Unfortunately, because of false-positive and false negative results due to antigen cross reactivity and weakening of specific immune responses due to disappearance of antibodies in long-term, these tests are unreliable for detection of carrier cattle (Passos et al. 1998). Therefore,

a sensitive and specific method as compared to serological tests and examination of Giemsa-stained blood smears for the diagnosis of piroplasms is required. Molecular methods, with a high degree of sensitivity and specificity, have been developed to identify various *Theileria* species in persistently infected cattle (Altay et al. 2008).

In Iran, theileriosis is normally diagnosed with the traditional Giemsa staining method, yet it seems not to be applicable for identifying of the carrier animals (Azizi et al. 2008). The aim of this study was to compare conventional Giemsa staining method with the PCR technique in the detection of *Theileria* organisms.

Materials and methods

Collection of blood samples

From June 2009 to October 2009, 30 farms in Isfahan province, central part of Iran, were selected for the study based on their history of outbreak of bovine theileriosis. Blood samples were collected from jugular vein of 150 Holstein and crossbred cattle. Five hundred micro liters of each collected blood samples was fixed with 1 ml 96 % ethanol in 1.5 ml sterile eppendorf tubes. Additionally, two thin blood smears by puncturing ear vein for each cattle were prepared. The blood smears were air dried, fixed in methanol, stained with Giemsa and analyzed for the presence of *Theileria* in the erythrocytes at 100× magnification. In each blood smear 50 fields were examined separately by a single observer. The presence of one piroplasm was considered as a positive blood smear. Microscopic examination was done in a manner that was blinded to the molecular results. All smears carefully examined to estimate the Percent Parasitized Erythrocytes (PPE).

DNA extraction

The DNA was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. Briefly, ca. 5 mm^3 big pieces of fixed blood samples were air dried and subsequently lysed in 180 µl lysis buffer and the proteins were degraded using 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 (96 %) was added to the solution and after vortexing, the complete volume was transferred to the MBST-column. The column was first centrifuged, and then washed twice with 500 µl washing-buffer. Finally, DNA was eluted from the carrier using 100 µl Elution buffer. The amount of extracted DNA and its purity was measured by OD260 and the ratio of OD260–OD280 respectively. In addition the extracted DNA was analyzed on agarose gel before use.

PCR

For assessment of the *Theileria* infection in samples, primers Tbs-S (5′CACAGGGAGGTAGTGACAAG3′) and Tbs-A (5′AAGAATTTCACCTCTGACAG3′) were used, which amplify simultaneously an approximately 426–430 bp fragment of the 18SrRNA gene for members of genera *Theileria*. Approximately 100 ng DNA was used for the PCR analysis. The PCR was performed in 100 µl total volume including one time PCR buffer, 2.5 U *Taq* DNA Polymerase (Cinnagen, Iran), 2 µl of each primer (Tbs-S/Tbs-A, 20 µM, Cinnagen, Iran), 200 µM of each dATP, dTTP, dCTP and dGTP (Fermentas, EU) and 1.5 mM MgCl2 in automated Thermocycler (MWG, Germany) with the following program: 5 min incubation at 95 °C to denature double strand DNA, 38 cycles of 45 s at 94 °C (denaturing step), 45 s at 55 °C (annealing step) and 45 s at 72 °C (extension step). Finally, PCR was completed with the additional extension step at 72 °C for 10 min. The PCR products were analyzed on 2 % agarose gel in 0.5 times Tris–Borate–EDTA (TBE) buffer and visualized using ethidium bromide and UV-illuminator. A molecular mass ladder (100 bp) and positive and negative controls were used for each batch run. Each sample was spiked with positive control *Theileria* spp. DNA to detect any inhibition of the PCR that might lead to false- negative results.

Statistical analysis

The degree of agreement between PCR and the microscopical tests was determined by Kappa (κ) values with 95 % confidence intervals. The PCR as the reference test was used to calculate the relative sensitivity and relative specificity of the microscopical tests.

Results

The DNA was extracted from blood samples and analyzed by PCR using primers derived from the 18S rRNA gene. The nucleotide sequence of 18S rRNA gene is highly conserved in *Theileria* spp. and the primers Tbs-S/Tbs-A can amplify the corresponding fragments of the gene in all known *Theileria* species.

The PCR analysis of the DNA isolated from blood samples showed that 42 out of the total 150 blood samples were *Theileria* spp. positive and revealed an expected PCR product of 426–430 bp in length (Fig. 1).

Interestingly, the Giemsa staining analysis of blood smears showed different results dependent upon the chosen number of examined microscopic fields by 100× magnification (Fig. 2). In 11 out of 150 blood samples, *Theileria* like structures could be identified when the examination

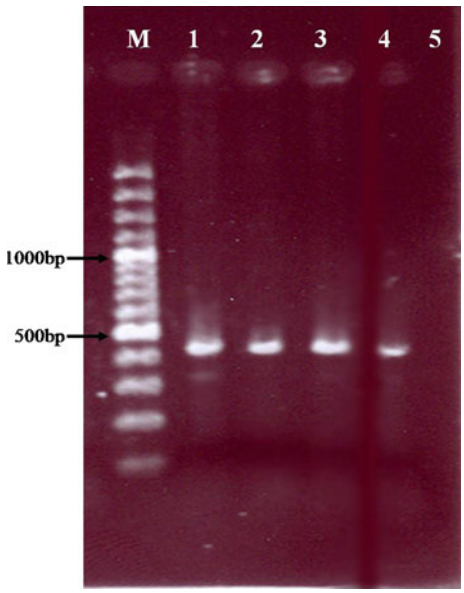

Fig. 1 Isolated DNA from blood was analysed by PCR. *Theileria* spp. positive control (*lane 1*), DNA was amplified with Tbs-S/Tbs-A primer set resulting in PCR product of 426–430 bp in length (*lanes 2–4*), negative control (*lane 5*) and *M* (Marker 100 bp)

was performed in 50 microscopic fields. Ten of samples (among 11 samples) showed also PCR positive (Table 1).

The percentage of erythrocytes harboring *Theileria* like structures varied in the positive blood samples from 10^{-3} to 5×10^{-1} %. The examination of 50 microscopic fields showed 57 % sensitivity and 99 % specificity compared to 100 % sensitivity and specificity for PCR (Table 2).

The κ coefficient between PCR and Microscopy (50 fields) indicated no level of agreement (−1.06).

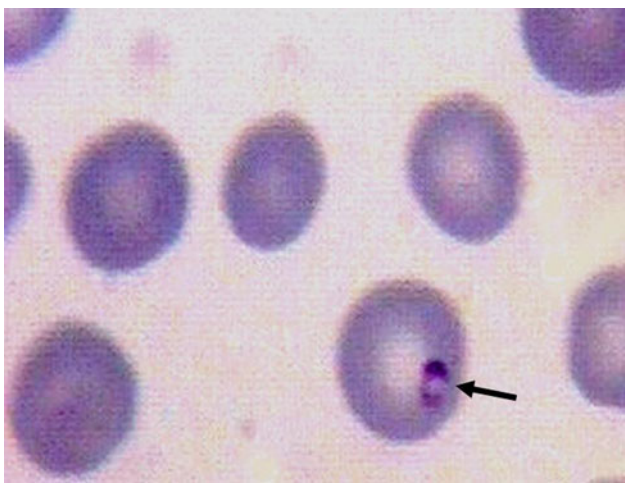

Fig. 2 Blood smear was analyzed by Giemsa staining. *Theileria* like structure is detectable in one of the erythrocytes (*arrow*)

Table 1 Comparison of results of PCR assay and microscopic examination for *Theileria* spp. in 150 cattle blood samples

PCR assay results	Microscopy results	
	50 fields	
	+	−
+	10	32
−	1	107

Table 2 Sensitivity and specificity of microscopical methods compared to 100 % sensitivity and specificity of PCR for detection of *Theileria* spp. in carrier cattle

Method	No. of samples examined	No. of positives detected	Sensitivity[a] (%)	Specificity[b] (%)
PCR	150	42	100	100
Microscopy (50 fields)	150	11	57	99

[a] Calculated as follows: [number of true positives/(number of true positives + number of false negatives)] × 100

[b] Calculated as follows: [number of true negatives/(number of true negatives + number of false positives)] × 100

Discussion

The Giemsa stained of blood smear is the most used method for the identification and characterisation of these piroplasms in Iran. This method accompanied with some technical problems cause false morphological diagnosis, and in some cases needs special diagnostic knowledge (Shayan and Rahbari 2005). PCR is now routinely used around the world for investigations of *Theileria* infections, particularly to determine carrier animals (Altay et al. 2005; Shayan and Rahbari 2005). The forward and reverse primers used in the present study was previously designed as a *Theileria* genus specific primer based on hyper variable region V4 of 18S rRNA gene sequences for the detection of this parasite in the carrier animals (Shayan and Rahbari 2005). Previously, the prevalence of *Theileria* infections in cattle was determined in central region of Iran using PCR (Noaman 2012) but sensitivity and specificity of microscopic technique in comparison with PCR is not evaluated yet.

Our results indicated no level of agreement between the microscopic examination and PCR. The examination of 50 microscopic fields showed 57 % sensitivity and 99 % specificity compared to 100 % sensitivity and specificity of PCR. With sole use of this method, 11 blood samples were identified as *Theileria* positive, from which 1 sample was false positive. This means that within 150 blood samples, 32 PCR positive samples were recognized as

false-negative. In Iran, the most of the veterinary laboratories examine 50 microscopic fields for detection of parasites in blood smears. Although this microscopic screening is specific, this approach often lacks the desired sensitivity. The detection of *Theileria* microscopically requires high parasitemia, good smear preparation, proper staining and a well-trained microscopist (in spite of the fact that the technique is cheaper and easier to perform). However, microscopic examination remains the convenient technique for day-to-day diagnosis of clinical cases in the laboratory but due to the very low amount (10^{-3}–5×10^{-1}) of infected erythrocyte is not able to detect carrier cattle (Salih et al. 2007).

Conclusion

The present study showed that the microscopic examination could fulfill the desired results for the diagnosis of acute Theileriosis but for the detection of carrier animal with low parasitemia, the PCR is preferable to microscopy with 50 fields. Our results showed that for the detection of cattle infected persistently with *Theileria* spp., the PCR can be used as a safe method and is recommended for epidemiological studies.

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