Bulgarian Journal of Veterinary Medicine (2012), **15**, No 2, 123–130

PREVALENCE STUDY OF *THEILERIA ANNULATA* BY COMPARISON OF FOUR DIAGNOSTIC TECHNIQUES IN SOUTHWEST IRAN

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Summary

Dehkordi, F. Safarpoor, P. Parsaei, S. Saberian, S. Moshkelani, P. Hajshafiei, S. R. Hoseini, M. Babaei & M. N. Ghorbani, 2012. Prevalence study of *Theileria annulata* by comparison of four diagnostic techniques in southwest Iran. *Bulg. J. Vet. Med.*, **15**, No 2, 123–130.

To identify the prevalence rate of *Theileria annulata*, blood and lymph node biopsy smears and blood and lymph node PCR have been developed. In 174 out of 1202 blood samples (14.478%) and in 129 out of 1202 lymph node biopsy samples (10.73%), the piroplasm forms and macroschizonts of *Theileria* were observed on blood and lymph node biopsy smears, respectively. A 230 bp band in both PCR tests that indicated the presence of *T. annulata* (prevalence rate) was present in 338 out of 1202 samples (28.11%). Statistical analysis showed significant differences ($P<0.01$) between the ability of biopsy smears and PCR tests to detect *T. annulata*. Our study entirely rejected the presence of the other *Theileria* species as parasitic pathogens in southwest Iran. After analysis of data, it was recognized that 14.47% and 8.23% of positive samples were on late and initial stages of disease, respectively. It was estimated that 32.24% of positive samples have been treated. Application of both PCR tests was more sensitive than the two other diagnostic assays to detect *T. annulata*. To our knowledge, this study is the first report of direct identification of *T. annulata* in blood and lymph node samples by evaluation of biopsy smears and PCRs tests in Iran.

Key words: blood PCR, blood smear, lymph node biopsy smear, lymph node PCR, *Theileria annulata*

INTRODUCTION

Theileria annulata is a tick-borne, obligate intracellular apicomplexan parasite causing tropical theileriosis in cattle, buffaloes and sheep (Ogre, 1999; Gubbels *et al*., 2000; Gubbels *et al*., 2002). Tropical theileriosis is difficult to control where the prevalence of vectors including certain Ixodid ticks such as fifteen *Hyaloma* spp. (Robinson, 1982), *H. detritum, H. anatolicum, H. excavatum* and *H. dromedarii* (Neitz, 1957), is high. The majority of cases occur between June and September (Flach & Ouhelli, 1992). In contrast to other tick-borne parasites such as *Babesia*, in the case of *T. annulata* a single tick is

capable to transmit a fatal infection (Pipano *et al*., 1982).

In spite of the importance of disease, there is no available effective drug for treatment or extensively used vaccine to control the disease. The methods currently used to protect against tropical theileriosis are expensive and all have serious limitations regarding their efficacy and sustainability.

Diagnosis based on the clinical signs is not trustful in many cases because other parasitic diseases have clinical signs similar to those of tropical theileriosis. The diagnosis of acute theileriosis cases is mainly based on microscopic examination of Giemsa-stained blood smears and lymph node biopsy smears. It should be outlined that the infection with the morphologically similar parasite, *T. buffali*, may confuse an accurate diagnosis of *T. annulata* (Barnett & Brocklesby, 1966). Antibodies to *T. annulata* can be detected by serological tests such as the indirect fluorescence antibody test (IFAT) but the IFAT lacks specificity due to cross-reactivity with other *Theileria* species and even *Babesia* (Burridge *et al*., 1974; Leemans *et al*., 1999). Other serological tests, such as ELISA, also lack either sensitivity or specificity (Gubbels *et al.,* 2000). Modern molecular techniques such as the polymerase chain reaction (PCR) allow researchers to study DNA directly.

This present investigation was carried out to study the prevalence of *T. annulata* in Holstein cattle by evaluation of blood and lymph node biopsy smears and blood and lymph node PCR in southwest Iran.

MATERIALS AND METHODS

Samples

Between June and September 2010, 1202 blood and lymph node biopsy samples were collected from Holstein cattles of 5 provinces in southwest Iran – Isfahan, Khozestan, Chaharmahal va Bakhtiary, Kohkiloye va Boyer Ahmad and Lorestan. Ten mL peripheral blood in EDTA-coated vacutainer tubes and lymph node biopsy samples were obtained from each animal. The samples were randomly collected from 92 dairy herds.

Light microscopic examination

Thin blood smears taken from the ear marginal vein to observe piroplasm forms and lymph node smears from superficial cervical (prescapular) lymph nodes to observe macroschizont (also known as Koch's blue body) stage were stained with 5% solution of Giemsa in buffer at pH 7.2) for 40 min and then examined at 1000× magnification (Benjamin, 1978).

DNA isolation

Genomic DNA was extracted from whole blood and lymph node biopsy samples using a DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The isolated DNA was quantified spectrophotometrically and on agarose gel prior to dilution to a final working solution of 20 ng/µL.

Analysis of samples with single PCR

To screen for *T. annulata*, PCR primers were designed on the basis of the sequence of the 18S ribosomal RNA gene (Accession No: EU083799, GenBank database, at NCBI). The forward primer sequence was 5'-TAGGGCTAATACATG TTCGAGAC-3', and the reverse primer sequence was 5'-ATAAGCCACAATGCA AAGACTC-3'. All oligonucleotide primers were obtained from a commercial source (Cinna Gen, Iran).

The PCR reaction was performed in a total volume of 25 µL containing 2 µL of

DNA sample, 0.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.8 µm of each primers and 0.5 U/reaction of *taq* DNA polymerase. Reactions were initiated at 94 °C for 5 min, followed by 30 cycles of 94 °C for 50 s, 58 °C for 50 s, 72 °C for 1 min and a final elongation step at 72 °C for 5 min, with a final hold at 4 °C in a DNA thermal cycler (Master Cycler Gradiant, Eppendrof, Germany). A negative control (sterile water), and a positive control DNA from a T. *annulata* strain, were included in each amplification run.

Agarose gel electrophoresis

Amplified samples were analyzed by electrophoresis (120 V/208 mA) in 1.5% agarose gel. Positive and negative PCR controls were run with each series of amplifications. The gel was stained with 0.1% ethidium bromide (0.4 µg/mL) and viewed on a UV transilluminator.

Statistical analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA), ANOVA tests were performed and differences were considered significant at values of $P<0.05$.

RESULTS

Blood and lymph node smears

Table 1 shows the prevalence of positive samples achieved with the four diagnostic methods. After studying Giemsa-stained blood smears under a light microscope, from a total of 1202 blood smears, 174 samples (14.47%) showed the piroplasm forms of *Theileria* including different shapes and abnormalities in erythrocytes (Fig. 1).

Table 1. Prevalence of *T. annulata* in 1202 samples collected from Holstein cattle from southwest Iran established by four diagnostic methods

Diagnostic technique	Number (%) of positive samples
Blood smear	174 (14.47)
Lymph node smear	129 (10.73)
Blood PCR	239 (19.88)
Lymph node PCR	164 (13.64)

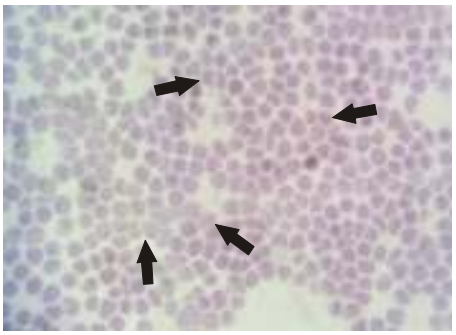

Fig. 1. Thin blood smear showing the piroplasm stage of the T. *annulata* including cocci, rod, stick, comma, fusiform, racquet-shaped, signet ring, and pear-shaped forms with diameter of 0.5–1.5 µm. Abnormalities in erythrocyte structure are also observed.

On Giemsa-stained lymph node biopsy smears, *Theileria* macroschizonts with several nuclei were observed on 129 (10.73%) out of all 1202 samples (Fig. 2).

Blood and lymph node PCR tests

The quality of extracted DNA after agarose gel electrophoresis was accepted as suitable for PCR assay.

After blood and lymph node PCR tests under the described conditions and electrophoresis, 239 out of 1202 blood samples and 164 out of 1202 lymph node samples, had a 230 bp band suggesting an

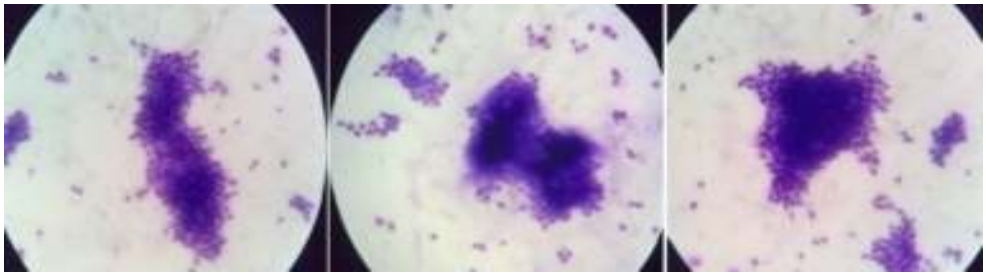

Fig. 2. Lymph node smear slides showing the macroschizont (also known as Koch's blue body) stage of *Theileria annulata*.

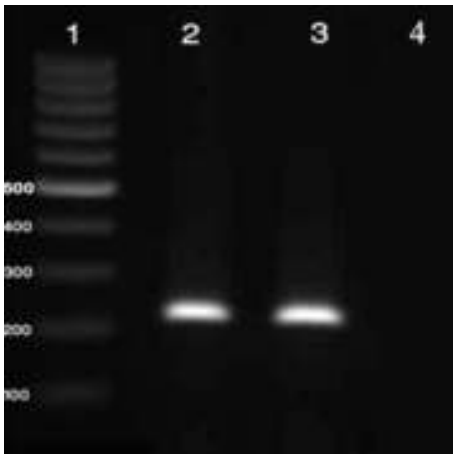

Fig. 3. Agarose gel electrophoresis of PCR to track the *T. annulata*'s gene in peripheral blood of Holstein cattle. 261 samples showed the 230 bp band. Lane 1 – 100 bp marker, lane 2 – positive control, lane 3 – test sample and lane 4 – negative control.

infection by *T. annulata* (Fig. 3) and therefore, the percentage of *T. annulata* positive samples were 19.88% and 13.64% in blood and lymph node samples, respectively.

The statistical analysis showed significant differences ($P<0.01$) between blood and lymph node PCR tests vs blood and lymph node biopsy smears for detection of *T. annulata*.

After comparing the results obtained from all four diagnostic methods, and to determine the true prevalence rate of *T. annulata*, 338 samples that were found to be positive by more than one diagnostic methods, had been finally declared positive for *T. annulata*. Therefore the prevalence rate of *T. annulata* in southwest Iran between June and September 2010, was 28.11%.

The prevalence of *T. annulata* in each of studied provinces was as followed: Isfahan – 21.97%; Khozestan – 65.83%; Chaharmahal Va Bakhtiary – 20.26%, Kohkiloye Va Boyerahmad – 57.44% and Lorestan – 30.86%.

Our study indicated that from a total 338 cattle positive for presence of T. *annulata*, 8.23% and 14.47% were in initial and late stages of disease, respectively and that 32.24% of them had been treated in the past (Table 2).

Table 2. Distribution of *T. annulata*-positive samples collected from Holstein cattle from southwest Iran (samples found to be positive by more than one diagnostic methods)

	Number (%)*
Total positive samples	338 (28.11)
Initial stage of disease	99 (8.23)
Late stage of disease	174 (14.47)
Treated animals	109 (32.24)

* Percentages are calculated vs the total number of 1202 samples.

DISCUSSION

T. annulata, which is the cause of tropical theileriosis, is an omnipresent parasite in Iran and has been reported practically from every part of the country (Hooshmand-Rad, 1977). The disease is very important in Iran and it was estimated that 250 million cattle in countries including Iran, Turkey, India and China are at a risk of the disease, which causes serious economic loss through bovine mortality and loses productivity (Campbell *et al*., 1999; Razmi *et al*., 2003).

The prevalence rate of theileriosis in southwest Iran (28.11%) in 2010 was higher than that established in the Kayseri province of Turkey (9.3%) (Ica *et al*., 2007) but lower than in Northern Spain (64.8%) (Nagore *et al*., 2004) and eastern Turkey (45%) (Aktas *et al*., 2006).

The differences between the prevalence of *T. annulata* among cattle of each province found in this study may be attributed to the different climate. Khozestan province has a hot and temperate weather beneficial for the development of Ixodid ticks living and this was probably the reason for the highest prevalence rate of *T. annulata* in southwest Iran.

In our study, the PCR diagnostic tests were found to be more accurate and sensitive than both microscopic assays. In a similar study, *T. annulata* detection through PCR test in blood samples of cattle was reported to be of higher efficacy (75%) compared to blood smear examination (22%) (Oliveira *et al.*, 1995).

No comments could be made about the superiority of one PCR method to another (blood PCR against lymph node PCR) as considering the life cycle of parasites, lymph node PCR assay is capable to detect *T. annulata* in initial stages of disease whereas blood PCR – at a later stage.

The blood smear assay detected T. *annulata* in 174 samples while blood PCR – in 239 blood samples (Table 1). A similar relationship was observed between positive lymph node biopsy smears (129) and positive lymph node PCR tests (164). Based on the life cycle of parasite, *T. annulata* at first infects lymph nodes and then passes into the bloodstream and red blood cells are also infected (Mehlhorn & Schein, 1984; Nalbantogiu, 2003). Therefore, samples that were reported positive by lymph node PCR method (118 samples), and negative by blood PCR assay (99 samples), were considered as early stage of disease. These cattle had been probably infected with *T. annulata* two or three days ago. Samples that were reported positive by blood PCR assay but were negative in the lymph node PCR test, were considered as late stage (chronic) disease (Table 2).

We were not able to detect parasites in samples that were negative by PCR tests. This fact, apart confirming the superiority of blood PCR and lymph node PCR vs blood and lymph node biopsy smears, rejected entirely the presence of other *Theileria* species in southwest Iran because all of samples with positive blood smear and lymph node biopsy smear were reported positive in blood PCR and lymph node PCR, respectively.

If animals suffering from tropical theileriosis are treated with antiparasitic drugs, *T. annulata* is removed from lymph nodes and remains in blood at very low numbers (Ahmed & Mehlhorn, 1999; Glass, 2001; Salama & Magdy, 2007). On the basis of our results, we could assume that from samples with positive blood PCR and negative lymph node PCR results (174 samples), 109 with negative blood smears (i. e. very low number of parasites in blood), could be considered as

treated cattle. Our results indicated that cattle, infected with *T. annulata* few days ago were less numerous than those infected long time ago, because the total number of samples positive by the blood PCR test was higher than those with positive lymph node PCR results. So it could be concluded that the number of cattle in the early stages of disease was lower than the number of those with intermediate and late (chronic) disease.

After the clinical examination of cattle with positive lymph node PCR and negative blood PCR results, it became clear that some of them showed the initial clinical signs of the tropical theileriosis including enlarged lymph nodes, fever, anorexia. Some of cattle with negative lymph node and positive blood PCR tests showed the clinical signs of late disease such as poor condition, diarrhoea, pale mucous membranes, jaundice, anaemia and sometimes haemoglobinuria; these findings came also in support of our claim. They also showed that application of both blood PCR and lymph node PCR methods allowed for a more accurate diagnosis than other tests for detection of *T. annulata* in cattle.

To our knowledge, this study is the first prevalence report of *T. annulata* by evaluation of blood smear, lymph node biopsy smear, blood PCR and lymph node PCR in Holstein cattle in Iran. It confirmed that despite all precautions, this infectious disease was highly prevalent in some parts of the world like Iran.

In conclusion, the higher accuracy of both blood and lymph node PCR tests to detect *T. annulata* compared to blood smear and lymph node biopsy smears, suggested that the simultaneous use of these two diagnostic methods (blood and lymph node PCR), can detect parasites in the early and late (chronic) stages of the disease. After comparing the results from all four diagnostic methods, 338 (28.11%) samples that were found to be positive by more than one diagnostic methods, represented the prevalence rate of *T. annulata* in southwest Iran between June and September 2010.

ACKNOWLEDGMENTS

The authors are thankful to the Veterinary Administration of the Chaharmahal Va Bakhtiary province and the managers of all herds in southwest Iran for their help to collect the samples.

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Paper received 01.10.2011; accepted for publication 20.01.2012

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