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Detection of *Theileria annulata* by PCR and its Comparison With Smear Method in Native Carrier Cows

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Abstract: During March and April 2007, 140 native cows with the mean age of more than one year were selected randomly and their age and sex were registered in the related papers. Primarily, a thin layer smear was prepared from their ear sublime vein blood and was fixed with methanol and then it stained with Gimsa dye. Also, 9 mL blood from the jagular vein of the same cow was taken and collected in autoclaved tubes (containing 1 mL of 3.2% buffered citrate solution) conversely for extracting their DNA for PCR. Two primers were used: N516/N517 belonged to a huge gene (30 kDa) which is responsible for coding the surface antigen of *Theileria annulata* merozoite. By detection of 140 prepared blood smear with light microscope and lens 100, piroplasmic forms of *Theileria annulata* were seen in 12 smear (8.57%). By PCR method, 56 DNA of *Theileria annulata* was separated from 140 samples (40%). Statistical comparison of PCR and smear methods in diagnosing of *Theileria annulata* carriers can explain a significant difference between these two methods ($p<0.05$). The results of this study shows that sensitivity and accuracy of PCR method in diagnosing of *Theileria annulata* carriers is more than common method of smear preparation and can be used in epidemiological studies for the sake of controlling, prevention and determining of immunological condition of cows in region.

Key words: Native carrier cows, PCR method, Smear method, *Theileria annulata*

INTRODUCTION

Theileriosis due to *Theileria annulata* which is also known as Mediterranean or tropical Theileriosis has vast geographical spread and can be found especially in Middle East, Mediterranean Europe, north of Africa, India, middle Asia and even China (D'oliveira *et al*., 1995).

In Iran, the native cows are more defensive to Theileriosis and are affected by subclinical form of the disease while the European cows are very sensitive to Theileriosis and if they do not treat effectively; their mortality rate will vary between 40-60% (Hashemifesharaki, 1991).

The treated cows and the native cows are carriers for a long period and even until the end of life that within this period of time, only few number of erythrocytes are contaminated with the parasite which their observation and also demonstrating of their presence can be done hardly (Kirvar *et al*., 2000). These chronic carries are the major agents of spreading the infection and have the most important role in alternation of the parasite life cycle between cows and tick (Ilhan *et al*., 1998).

Observation of *Theileria annulata* piroplasmic forms by smear method is the common way of diagnosing the contaminated cows and also is the oldest test for diagnosing this parasite in Iran. Smear method is associated with technical problems and even wrong diagnosing and has low sensitivity in diagnosing the carrier cows (Shayan and Rahbari, 2005).

Diagnosing of piroplasmic forms of *Theileria annulata* in carrier cows is an important epidemiologic parameter (D'oliveira *et al*., 1995). Accurate diagnosing of the carriers is necessary for determining the immunological condition of the animals and also controlling programs (Ilhan *et al*., 1998). Frequently serological methods are employed in determining subclinical infections. False positive and negative results are commonly observed in serological tests due to cross-reaction, weakening in specific immune response as well as lack of determination of antibodies in carriers because of long term infection (Leemans *et al*., 1999; Gubbels *et al*., 2000).

The PCR method is more accurate in comparison with the Immune Florcent Antibody (IFA) test, smear method and microscopic detection of piroplasmic forms. Because the PCR method has its superiority in separating parasitic infection associated with clinical signs (clinical form) and the infection without clinical signs (the subclinical form) (D'oliveira *et al*., 1995; Almeria *et al*., 2001).

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Kirvar *et al.* (2000) has reported that the sensitivity of this method is diagnosing one parasite per mL of blood and the accuracy of it is three times more than detection of parasite by smear method in microscopic field 200. D'oliveira *et al.* (1995) has demonstrated that the lowest extent of PCR *Theileria annulata* carrier diagnosing 2-3 parasite in blood unit that this amount is equal to 0.000048% of parasitemia. This study was undertaken to report the number of carrier native cows in Najaf Abad abattoir and the usage of the PCR method in diagnosing these carriers in compare to smear method and also determining the immunological condition of the cows in this region.

MATERIALS AND METHODS

During March and April 2007, 140 native cows with the mean age of more than one year were selected and their age and sex were registered in the related papers. Primarily, a thin layer smear was prepared from their ear sublime vein blood and was fixed with Methanol and then it stained with Gimsa dye. Also, 9 mL blood from the jagular vein of the same cow was taken and collected in autoclaved tubes, containing 1 mL of 3.2% buffered citrate solution (Barker *et al.*, 1992). Then these blood tubes were transferred to the laboratory and their *Theileria annulata* DNA extraction was performed according to the method described by D'oliveira *et al.* (1995) and Clausen *et al.* (1998) and stored at -20°C until analyzing. The thin layer smears of blood were stained by Gimsa dye within 40 min. Afterwards, prepared blood smear was seen with light microscope and lens 100X for the detection of *Theileria annulata* piroplasmic forms.

One set of primers was used (Table 1): N516/N517. To detect the PCR fragments generated by primer set N516/N517, that containing the complete cDNA encoding the 30 kDa major merozoite antigen was used as a probe. (D'oliveira *et al.*, 1995; Dumanli *et al.*, 2005).

To prepare TBE (0.5 X) buffer, 450cc distilled water is mixed to 50 mL TBE (0.5 X), 1/2% gel is used to prepare the gel 10 mL autoclaved TBE (0.5 X) buffer is extended to 100 mL with distilled water, then 0.9 g agarose gel is added, 75 mL TBE (0.5 X) buffer.

Table 1: Oligonucleotide primers used to amplify 30-kDa of *T. annulata*

Primer	Sequence[a]	Position[b]	Characteristic
30 kDa gene			
N516	GTAACCTTTAAAAACGT	234-250	*T. annulata* specific
N517	GTTACGAACATGGGTTT	954-938	*T. annulata* specific

[a]The 5'-3' primer sequence is given

3.75 microliter ethidium bromide is added to the gel it melted completely. ethidium bromide makes the DNA visible under UV rays.

We transferred the product into agars wells, then the Trans illuminator was connected to the electrophoreses tanks, in 85 V, DNA parts were separated in 2.5 h.

RESULTS AND DISCUSSION

In detection of 140 prepared smears, 12 of them had shown piroplasmic forms of *Theileria annulata* (8.57%). While in the other 128 samples, these piroplasmic forms couldn't be found so that, they were considered negative. PCR reaction was done on extracted DNA of 140 blood samples and amplified products were separated by electrophoresis on a 1% agarose gel, then the results were analyzed. The amplified sequence weight by N516/N517 primers from the extracted DNA was 721 bp. This weight was determined by the weight of another marker which was separated with other products of PCR on electrophoresis gel and was 100 bp DNA leader. The molecular weight (721 bp) of the desired *Theileria annulata* sequence which is synthesized by N516/N517 primers (Meta Bion Co.) is in the marker limit weight (700-800 bp). Amplified DNA sequences were positive and showed *Theileria annulata* and were negative out of this limit (Fig. 1).

In 56 samples of 140 examined samples, the desired sequence with the weight of 721 bp was seen which considered as contamination with *Theileria annulata* (40%). In 84 samples, we could not find any amplified sequence in the desired limit weight (Table 2).

The statistical comparison of PCR method and smear preparation in diagnosing the *Theileria annulata* carriers by Mcnemar statistical test has shown a significant variation between these two methods ($p<0.05$).

Performing affected programs for prevention and controlling *Theileria annulata* depends on accurate diagnosis of contamination in cows and infected ticks.

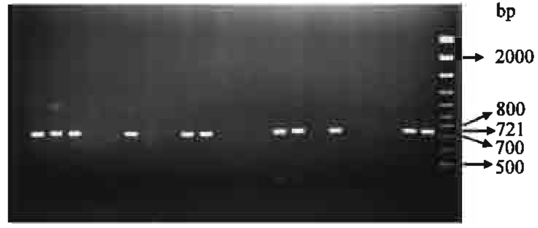

Fig. 1: Agarose gel electrophoresis of amplified DNA by using primer set N516/N517, which is coding the merozoite surface antigen of *Theileria annulata*

Table 2: Comparing the two methods of PCR and Smear preparation in diagnosing the *Theileria annulata* carriers

	Diagnosis methods			
	PCR		Smear	
Test results	No.	(%)	No.	(%)
Positive	56	40	12.00	8.57
Negative	84	60	91.43	91.43
Total	140	100	100.00	100.00

The native cows are defensive to *Theileria annulata* and are affected by subclinical form of the disease which these cows will be carriers until the end of their life. In carriers, only the piroplasmic forms of the parasite can be observed hardly (Kirvar *et al*., 2000). The native carrier cows are the major agents of spreading the infection and have the most important role in alternation of the parasite life cycle between cows and tick. The accurate diagnosis of carriers is necessary for determining the immunological condition of the animals and prevention of the disease (Ilhan *et al*., 1998).

In this study, the results of PCR method showed that 56 of 140 cows with more than 1 year age were carriers of the parasite *Theileria annulata* (40%). These results show the high rate of native cows contamination with *Theileria annulata* in the form of subclinical or carrier also, they show the immunological condition of the native cows in region. The PCR accuracy in diagnosing carriers in comparison with smear method had been seen in 12 samples which showed the piroplasmic forms of the parasite. This also can show the necessarily of using this method in epidemiological studies, determining the immunological condition of the animals and performing prevention and controlling programs.

D'oliveira *et al.* (1995) contaminated 92 one-year-old cattle of Bostaurus breed with four strains of *Theileria annulata* which were collected from Turkey, Spain, Portugal and Mauritania and tried to diagnose this experimental contamination by PCR, IFA and Smear and also compared these three methods. The positive cases in Smear, IFA and PCR methods were 22, 40 and 70%. Roy *et al.* (2000) reported that in detection of 50 blood samples of native cows by PCR and Smear methods, the positive cases by PCR were 42 and by Smear method only 8 of them were positive. Dumanli *et al.* (2005) in detection of 1561 blood samples of various cows breeds which were collected from east region of Turkey, used PCR, IFA and Smear methods for diagnosing the *Theileria annulata* contamination. The positive cases in Smear, IFA and PCR methods were 590, 526 and 293. Martin-Sanches *et al.* (1999) in detection of 214 blood samples in Spain using PCR, IFA and Smear methods for diagnosing the *Theileria annulata* contamination, had shown that PCR method had more accuracy and sensitivity in comparison with the Smear method. Almeria *et al.* (2001) used two methods of PCR and Smear conversely for epidemiological study of native cows contamination to *Theileria annulata* in Minoyca region and reported that by using the PCR method, 48.3% of the cows had contamination, also had shown that PCR method had more accuracy and sensitivity in comparison with the Smear method.

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