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Molecular Survey of *Theileria annulata* in Cattle by PCR - RFLP Method in Iran

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Abstract

Theileriosis is one of the infectious diseases of cattle and buffalo in tropical countries and Mediterranean region that is of a considerable economical value, generated by *Theileria haemoprotozoan* and transferred by vetors belonging to Ixodidea. The present study investigates 52 samples in west and east Azerbaijan, Sanandaj and Kermanshah in Iran (2009). In this study, *Theileria annulata* was separated from ill and healthy cattle by PCR-RFLP method. Especial primers were achieved from *Sml-2* gene for this procedure. A fragment of 270bp was separated from the gathered blood samples. PCR products were distinguished by agarose gel and ultraviolet rays system. Among 22 naturally infected cattle, 13 cases (59.09%) were positive in blood smear and 18 (81.81%) were positive in PCR. Among the random sampled healthy cattle, from 30 cases, 4 cases (13.32%) were positive in blood smear and 5 (61.66%) were positive in PCR. In order to investigate the genetic variation (PCR-RFLP), the separated 270bp fragment was digested by restriction enzymes such as *TaqI, BSURT, AIUI, RsaI. TaqI* enzyme generatd (200, 170bp) fragments, AIUI enzyme generated (120, 90, 60bp) & (150, 90, 30bp) fragments and *RsaI & BSURI* enzymes could not digest the considered fragment. Finally, six different patterns in genetic variation of relevant gen were observed.

Keywords: Gene; *Theileriosis*; Cattle; PCR; Blood; Enzyme; Base pairs

Introduction

Theileriosis resulting from *Theileria annulata* in cattle is an important, dangerous and fatal parasitical disease having great economical value [1,2,3,4]. *Theileria* spp infects domestic and wild ruminants in tropical and subtropical regions in the world [5]. Also, it is a piroplasma protozoa of cattle and buffalo called tropical fever made by a kind of *Theileria* such as *annulata* [3].And causes Lympho-proliferation disease, which is a high rate mortality disease [6]. *Theileria annulata* infects lineage cells of macrophage/monocytes and B Lymphocytes in cattle [7,8]. According to the evaluations, more than 200-250 million cattle and calf are at the risk of theileriosis all around the world [9]. Some important clinical signs of this disease are inflation of prescapular and prefemoral lymphatic glands, fever, diarrhea, anorexia, anemia of eye & vagina mucus, jaundice and outpouring of nose water [9-13]. *Theileria annulata* is one of the fatal Theiloriosis in Europe, North Africa and Middle East [14]. In a study on 680 ticks taken from 107 infected cattle by *Theileria annulata*, the results were as follows: 92/35% (*Hyalomma anatolicum anatolicum*), 5/14% (*Hyalomma anatolicum excevatum*), 1/17% (*excevatum excevatum*), 1/32% (*Rhipicephalus Sang*) [3,12,15]. *Theileria annulata* does most of the cattle infection by ticks in Iran causing the death of domestic animals. The first report was announced in 1935 transferred by *Hyalomma excevatum* and *H. Detritum* [3,15,16]. The researchers observed infected materials called "Koch blue body" in lymphatic glands, liver and spleen of affected cattle that is the schizont stage of parasites. In north part of Iran, especially in North West and some parts of the west where the activity season is short due to having long winters [3]. Several studies and reports of this parasite are available. The first study was done by Rachel Adamson et al. [17] titled "Transient transfection of *Theileria annulata*". The second one has been done by Schinittger et al. [5] named "sequence analysis of *Theileria Lestoquardi* and a *Theileria* species highly pathogenic for small ruminants in China. Heidarpour Bami et al. [18] research was Molecular identification of ovine *Theileria* species by a new PCR-PFLP method. In 2008, Kursat Altay et al. [19] conducted a study named molecular detection of *Theileria* and *Bebsia* infections in cattle. Distinction accomplishes in regard to season, background and clinical signs. But the exact distinction of this organism is necessary for its classification and epidemiology. At first, the existence of *Theileria* would be definite by preparing blood smear and aspiration from inflated lymphatic gland and observing parasite in RBC or schizont [15,17]. Serological test, indirect immunofluorescent test and also the carriers would be distinguished by PCR [19,20]. Several successful studies and reports of cattle's PCR have been done [1,21-23].This molecular technique reveals a high sensitivity compared to monological examination and serological testing [24]. The progress in molecular biology can prove genotopic and useful characteristics for recognition and classification of numerous kinds of homoparasite from *Theileria* group [25]. By PCR, *Theileria* can be identified from *Anaplasmasis* [26], *Babesiosis* [27], *Leptospirosis* [3], *Leucosis* [28] and brain form of *theileria* [12]. PCR is a revolutionary method developed by Kary Mullis in 1980 [15,24]. Polymerase Chain Reaction (PCR) is a biological and molecular technique for DNA enzymatic transcription without using live organism such as E. coli and usually is used for medical and biological investigations for different purposes. PCR is used for small fragment duplication of DNA string and it can be a single gene or part of a gene [29]. PCR process has 3 phases, which includes 20-35 cycles, and each cycle has three parts

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[24,30]. The present study investigates molecular survey of *Theileria annulata* in cattle by PCR_RFLP method.

Matherials and Method

Field study area

The study was conducted on 52 cattle in the provinces of west Azerbaijan, East Azerbaijan, Kermanshah and Sanandaj located in the North West of Iran (2011) (Figure 1). This area has varying weather conditions. Unlike the dry and hot East and Central Iran regions, a climate region with rainfall and mild temperatures with warmer summers and colder winters.

Experimental approach

In 2011, during a period corresponding to the season of tick activity, every cattle suspected to *Theileriosis* was clinically examined. Then, blood smears were prepared from jugular vein. Also, the whole body of animal was inspected for the presence of ticks; the ticks removed were put into labelled flasks. They were kept cool and humid during return to the laboratory for further identification and examination

Collection of blood samples, blood smears and microscopic examination

Blood samples (stored at 20°C until DNA extraction and gathered randomly. The age of animals ranged between 5-6 months and 12 years old, and all were clinically healthy (None of the cattle had received treatment or a vaccination to block theileriosis.) were collected (The procedure of gathering samples took three continuous months) in EDTA from cattle exposed to *Ixodid ticks* in four geographical areas in Iran. Blood (All blood samples were collected from a jugular vein into anticoagulant EDTA bottle, about 5 ml bloods was taken from jugular vein of cattle) was used to prepare thin blood smears for microscopic examination and to extract DNA for PCR analysis. Then transferred to Alsiver environment the sample was immediately put into refrigerator. Blood smears were air-dried, fixed with methanol for five min, stained in 10% Giemsa at a dilution of 5% in buffer solution for 30 min, and then examined for the presence of *Theileria annuluta* under light microscopy.

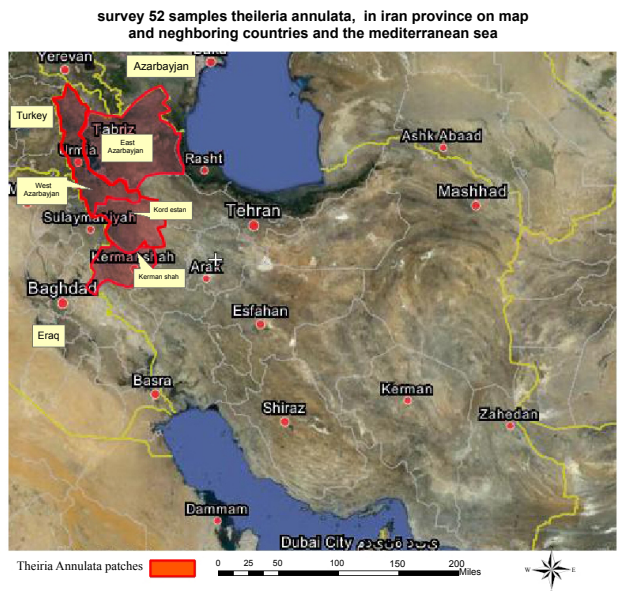

Figure 1: Surveying of 52 samples of Theileria annulata in iran provinceon map and neighboring countries and the Mediterranean sea.

DNA extraction

DNA extraction was performed as described by Lennette method. Briefly, 200ml of blood was added to 500 ml of lysis solution (0.32 Msucrose, 0.01 MTris, 0.005 M MgCl, 1% Triton X-100, and pH 7.5). The mixture was centrifuged at 13000×g for 5 min. The pellet was washed three times by centrifugation with500ml lysis buffer. The final pellets were resuspended in 100 ml of PCR buffer (50 mM KCl, 10 mM Tris–HCl and pH 8.3), 0.1% Triton X-100, pH 8.3). Proteinase K (50 mg/ml) was added to the pellet suspension, and the mixture was incubated at 56.8°C for 1 h. Finally, the samples were heated at100.8°C for 10 min.

Primers designed and PCR

For PCR amplification of *Theileria annulata*: One set of primers was used to amplify an approximately 270bp fragment of the Sml-2. Outer primersfor the primary PCR were forward strand primer *Thei* F1:5'_TGT-CAA-GGA-GGC-CTC-AAA-TT-3' and reverse strand primer *Their* F21:5'_TTT-GAC-TTT-GAA-TAG-GGT-GC-3'

Were used as described by Schnittger et al. [5]. For differentiation of various *Theileria* restriction fragment length polymorphism (RFLP) of PCR products of the *Sml-2* of *Theileria annulata* was done. The PCR amplified a monomorphic DNA fragment of 270 bp (Fermentas, Germany) which was sequenced and analyzed for the presence of restriction sites that differentiated the one haemoprotozoan in the presence of the host DNA. The enzymes *TaqI, BSURT, AIUI and RsaI*. *TaqI* were used. The amplified products were digested with restriction enzymes (Fermentas) as described by the supplier, and analyzed by agarose gel electrophoresis on a 2% gel. The digestion reaction was set up in 20µl volumes in 500µl PCR tubes. 10µl of the PCR product was used for each digestion. The digestion mixture consisted of 2µl of the 10× buffer, 10µl PCR product and 1µl (10U) of the restriction enzyme made up to 20µl with autoclaved triple-distilled water. The digestion mix was incubated at 37°C for 2 h. The restriction enzyme analysis of the PCR-amplified DNA fragments was performed by electrophoresis on the ethidium bromide-stained 2% agarose gel. .the gel; we cut off the electricity and transfer the gel to the UV transaulv mynatur to be observed. While working with Ethidium bromide we must use gloves and mask and after observing the gel in UV, and transfer the gel to the dangerous garbage section.

Restriction digestion

Twenty microlitres of amplified DNA from the PCR reaction were subjected to restriction enzymes digestion using *TaqI, BSURT, AIUI,* and *RsaI*. According to manufacturer's instructions (Promega, USA) and visualised by agarose gel electrophoresis (Table 1).

Results

Among 52 blood samples, 19cases of them were from west Azerbaijan, 3cases from veterinary clinic of Tabriz University having some clinical signs and 30 cases were randomly chosen from Sanandaj and Kermanshah from healthy and suspicious cattle. The result of the blood smear is as below: 1) West Azerbaijan and clinic: 13 positive cases and 9 negative cases. 2) Sanandaj and Kermanshah: 4 positive cases and 26 negative cases. Positive numbers of west Azerbaijan and clinic: (1, 2, 4, 7, 8, 9, 10, 11, 13, 14, 17, 18, 19), Positive numbers of Sanandaj and Kermanshah: (1, 12, 5, 52) (Figure 2 and 3).

Enzyme	Cut	Buffer	Thermal Digestion	Inactivation
TaqI	5' …TGGA … 3' 3' …AGCT … 5'	Unique	65°C	2/0L of EDTA 0/5M
BSURI	5' …GCCC … 3' 3' …CCCG … 5'	R	37°C	80°C for 20 min
RSaI	5' …GTAC … 3' 3' …CATG … 5'	Tango	37°C	65°C for 20 min
AluI	5' …AGCT … 3' 3' …TCGA … 5'	Tango	37°C	65°C for 20 min

Table 1: Twenty microlitres of amplified DNA from the PCR reaction were subjected to restriction enzymes digestion using *TaqI, BSURT, AIUI*, and *RsaI*. According to manufacturer's instructions (Promega, USA) and visualised by agarose gel electrophoresis.

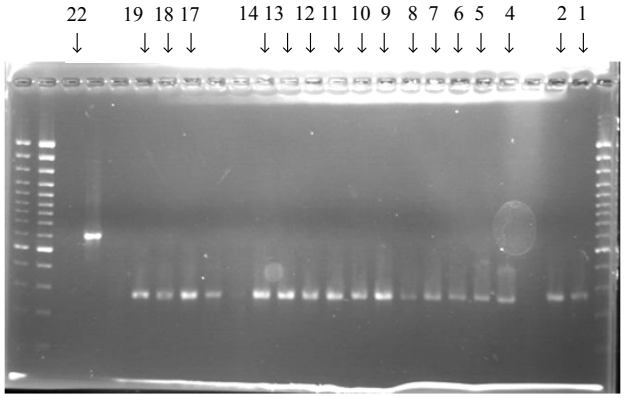

Figure 2: Positive samples of West Azerbaijan and clinic, Sample 22 is positive control and the first and last two ones are marker 100bp, Voltage is 150V, gel density is 1% and running time is 1/35 hours.

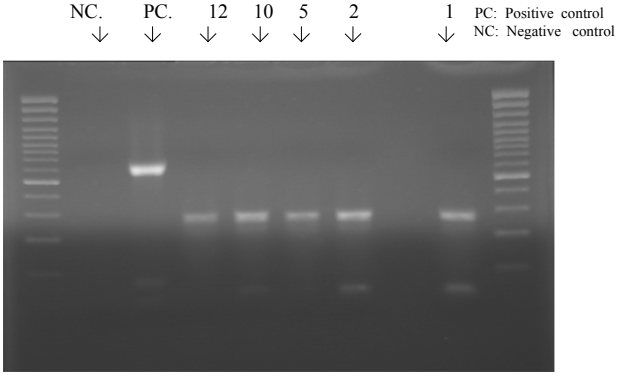

Figure 3: Positive samples from Sanandaj and Kermanshah, the first and the last marker 100bp.

The result of PCR on blood samples

By doing PCR on blood samples of west Azerbaijan and clinic, among 22 of collected samples from 12 age cattle and 5-6 months calf which had clinical signs of Theileriosis, 18 cases were positive and the size of separated fragment was 270bp (22, 16, 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 19, 17, 18). While there were only 5 positive cases in Sanandaj and Kermanshah (1, 2, 5, 10, 12). Also, in this case, the size of separated fragment was 270bp. (Table 2,3 and 4) The results of genetic diversity of *Theileriosis annulata* samples by enzymatic digestion of PCR products (RFLP-PCR) and positive samples of PCR products by using *TaqI, BSURI*, and *AIUI* enzymes were examined and the results were as follow:

Sample no.	smear	PCR
1	+	+
2	+	+
3	-	-
4	+	+
5	-	+
6	-	+
7	+	+
8	+	+
9	+	+
10	+	+
11	+	+
12	-	+
13	+	+
14	+	+
15	-	+
16	-	+
17	+	+
18	+	+
19	+	+
20	-	-
21	-	-
22	-	+

Table 2: The results of samples gathered from Kermanshah & Sanandaj.

Sample no.	smear	PCR
1A	-	-
2A	+	+
3A	-	-
4A	-	-
5A	-	+
6A	-	-
7A	-	-
8A	-	-
9A	-	-
10A	+	+
11A		-
12A	+	+
	-	-
30A	-	-

Table 3: The results of samples gathered from East & West Azerbaijan.

		Province	n		+	%
Blood smear Test T	With clinical signs	East & West Azerbaijan	22	9	13	59/09%
	Without clinical signs	Sanandaj & Kermanshah	30	26	4	13/33%
Total statistics of 52 samples in blood smear	With clinical signs & without clinical signs	All provinces	52	35	17	32/69%
n	Number of samples					
+	Positive samples					
-	Negative samples					

Table 4: Investigating the results of blood smear and PCR in samples from neighboring provinces of Turkey, Iraq & Azerbaijan Republic in Iran.

A) The results of digestion by *TaqI* enzyme: From 23 positive case, 11 of them were not digested (270bp), 3 cases were 70 & 200 and 9 cases were 100, 170. This shows two different places for *TaqI* enzyme in *Theileria annulata* DNA (Table 5 and Figure 4).

B) Digestion results of *BSURI* enzyme: No sample was digested among 23 samples (Figure 5).

Sample No.	Digestion with/by TaqI	Sample No.	Digestion with/by TaqI
1	100,170	13	170,100
2	200,70	14	170,100
4	200,70	16	170,100
5	200,70	17	270
6	270	18	270
7	170,100	19	270
22	270		
8	270	A1	270
9	170,100	A2	270
10	170,100	A5	270
11	170,100	A10	270
12	170,100	A12	270

Table 5: Digestion results by TaqI (samples 1 to 22 are related to west Azerbaijan & clinic and samples A1 to A12 are related to Sanandaj & Kermanshah.

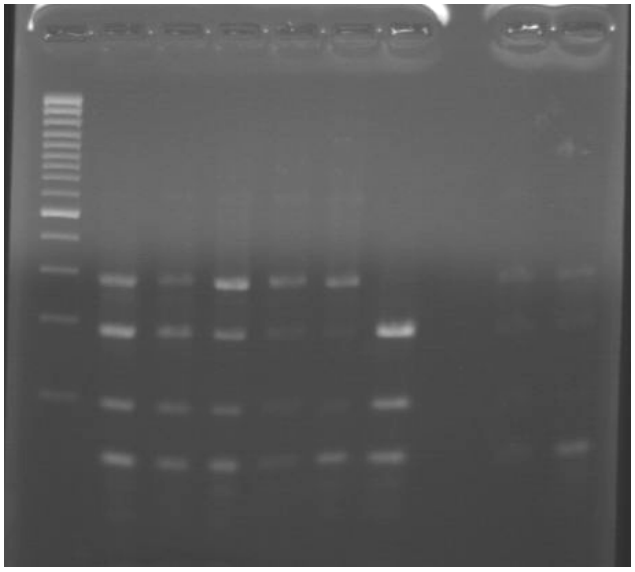

Figure 4: Enzymatic digestion by Taq 1.

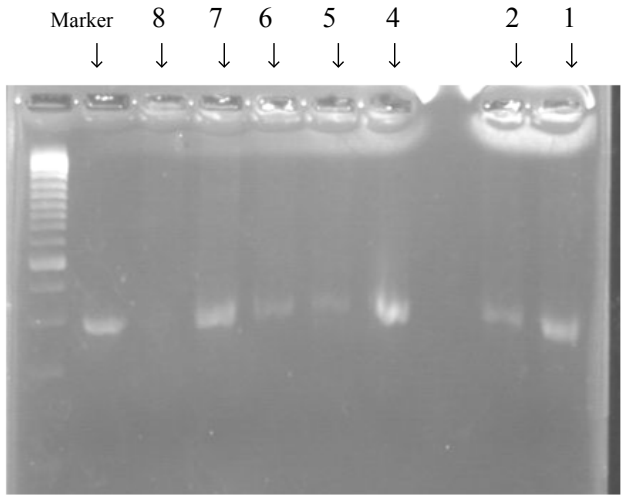

Figure 5: Samples not digested by BSURI enzyme.

C) The results of digestion by *RsaI*: Among 83 positive samples, none of them were digested by RsaI.D) the results of digestion by *AIUI*: among 23 positive samples, 7 cases (270bp) didn't digest, 14 cases were (60, 90, 120) and 2 cases were (30, 90, 150). This shows two different places for *AIUI* enzyme in *Theileria annulata* DNA (Table 6).

Abstract of PCR results

By doing PCR on blood samples the following results were obtained.

(a) We could separate *Theileria annulata* (270bp) by doing PCR with special primers.

(b) In blood smear of cattle having clinical signs of *Theileriosis*, 13 cases were positive out of 22 (59/09%).

(c) In PCR, 18 cases were positive among 22 cases (81/18%).

(d) In blood smear of cattle having no clinical signs, 4 cases were positive among 30 (13/33%).

(e) In PCR, 5 cases were positive among 30 cases without having any clinical sign (66/16%)

(f) Totally, among 52 healthy and ill cattle, 17 cases were positive in blood smear (32/69%)

(g) Among 52 cases, 23 cases were positive in PCR (44/23%).

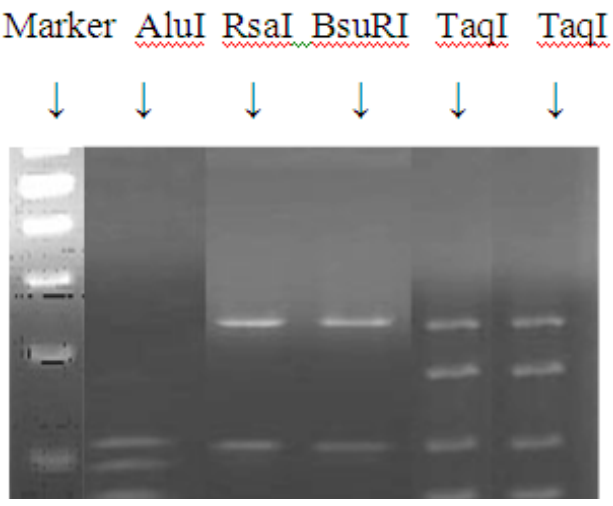

Figure 6: The results of enzymatic digestion by Taq 1 (170, 100), BSURI (270), RsaI (270) and AIUI (60, 90, 120) enzymes.

Sample No.	Digestion by AIUI	Sample No.	Digestion by AIUI
1	120,90,60	13	120,90,60
2	270	14	150,90,30
4	270	16	120,90,60
5	270	17	120,90,60
6	270	18	120,90,60
7	270	19	120,90,60
22	120,90,60		
8	120,90,60	A1	
9	120,90,60	A2	120,90,60
10	120,90,60	A5	270
11	120,90,60	A10	270
12	150,90,30	A12	170,100

Table 6: Digestion results by AIUI.

An abstract on the results of surveying genetic variation with enzymatic digestion. By using four DNA digesting enzymes, 6 different patterns were diagnosed in genetic variation of *SmI-2* of *Theileria annulata*.

Discussion

Theileriosis is an important and dangerous parasitical disease resulted from *Theileria annulata* and transferred by different kinds of ticks belonging to *Hyalomma*. This disease is seasonal, starts in the second part of April, adds to its abundance gradually and this abundance increases in June and July but in August and September the number of affected cattle is reduced by temperature decreasing and ticks' activity [32]. According to the estimates, about 200 million cattle and calf are at the risk of Theileriosis resulting from *Theileria annulata* and about 9 million of them are perished annually [9]. According to Christine study *Theileria annulata* is a protozoan parasite of domestic cattle and buffalo (Babalus Babalus) transferred by *Hyalomma ticks*. This disease is spread in North Africa, South Europe, India, South Russia and Middle East and contains 90% death rate in non-aboriginal races, and %5 and less in aboriginal races [32]. Cattle with subclinical *Theileriosis* become chronic carrier of the piroplasm and, hence, sources of infection for tick vectors. Therefore, latent infections are important in the epidemiology of the diseases. The diagnoses of piroplasm infections are based on clinical findings and microscopic examination of Giemsa-stained blood smears [22]. In this period, erythrocytes infected by piroplasma *Theileria annulata*. In this situation, finding *Theileria annulata* in blood smear is very difficult. On the other hand, under field condition, the diagnosis of Theileriosis resulting from *Theileria annulata* or *Theileria* mutans that have low pathogenetic is important and it is impossible to distinguish *Theileria annulata* from other kinds of *Theileria* [32]. By the invention of PCR method, sensitivity diagnosis test has improved [32]. According to the study done on *Theileria annulata* by PCR method using special primers of main antigen of the surface of merzoite *Theileria annulata* (30-kDa), the specialty of the procedure in non-duplicating DNA of the *theileriosis* except *Theileria annulata* (such as *Theileria pavra*, *Theileria mutans* and *Theileria Sergenti*) and other blood parasites (such as *anaplasma marginale, anaplasma centrale, Babesia bovis and Bayjimna*) was confirmed. In this investigation, from 52 samples, 32% of them were positive in blood smear, 40% in IFA and 75% in PCR. This shows that PCR is a useful instrument for *Theileria* strain isolation with high security [32]. Another study investigates the prevalence of *Theileria sergenti* in aboriginal cattle by PCR method with a private primer, the 128bp fragment was reproduced. In this study, 300 cattle were investigated that %8 of them were positive in blood smear. Meanwhile, 67.8% was positive in PCR method. In addition, this study indicated that the amount of infection in female cows were 70.3% and 40% in males (p<0/01) and also the amount of *Theileria sergenti* in cattle older than 3 years old were 75% and 61.8% in cattle younger than 3 years(p<0/05). In this study, the amount of infection in pasture cattle was %71.6 and %51 in non-pasture cattle (p<0/01) [33]. In a research by Witkowski et al. [29] on *Theileria* carrier ticks in infected cattle by PCR and probe DNA, it was indicated that 56% of the ticks out of a hundred feeding from infected animals were positive in PCR method. Meanwhile in probe DNA 37% of them (ticks) were positive and in the ticks on carrier cattle, 28% of them were positive in PCR method and 1.33% were positive in probe DNA [29]. In a study on apparently healthy and afflicted cattle by PCR method using special primers (Sml_2), it was distinguished that *Theileria annulata* is dominant strains of *Theileria* in west Azerbaijan. In this investigation, among 22 infected cattle by *Theileria annulata* that were ill naturally, 59.09% of them were positive in blood smear and 81.81% were positive in PCR and from 30 healthy cattle in blood smear which was difficult to distinguish, 3.33% of cases were positive in blood smear and 16.66% were positive in PCR. This study is matched to Kristine et al. study & Kun-Ho Song and shows that PCR is a powerful instrument for *Theileria annulata* with high sensitivity and specialty and non of the previous methods are comparable to PCR method. Because of high specialty and sensitivity of PCR method, Tilhan et al. [31] did a study on the effect of Sialic acid in resistance infection. For the purpose of this study, they infected 16 cattle tentatively with *Theileria annulata*, their infection was confirmed by PCR method, and 13 cattle were selected by PCR as a negative control. The density of Sialic acid in control cattle was 1141/38±68/14µg/ml and was 252/09±47/95µg/m (p<0/01) in infected cattle. It was 1502/71±116/13µg/m (p<0/05) 3 months after the treatment in infected cattle that was higher than the control cattle. This showed that *Theileria annulata* increases Sialic acid and is indirectly effective in setting up and sustaining infection in hosts' body [34]. We can identify Eimeria in picogram (less than 10 oocytes) by PCR. Cryptosporidium is recognized in excrement by microscope when the number of oocyte is more than 50000 in excrement, While, *Cryptosporidium parum* is recognizable by 16 oocyte in PCR method [35]. PCR have many problems by its unique characteristics. Some of them are the high cost compared to the smear staining, the need to train experts and have special abilities for recognition [36]. Also, PCR is not able to recognize dead parasites from the alive. Furthermore, it may seem false positive because of the high sensitivity and unwanted DNA infection. Now, PCR is semi-quantity and vague test and is completely substituted instead of old recognition procedures in developed countries [37]. In genetic variation with restriction enzymes on 270bp fragment for *Theileria annulata* genome, the considered fragment was digested by restriction enzymes of *RsaI, TaqI, BSURI & AluI*. *TaqI* enzyme generated fragments (170, 100bp), (200,70bp), *AluI* enzyme generated fragments (60, 90, 120bp) and fragment (30, 90,150), *RsaI & BSUR* enzymes were not able to digest any of the samples. In total, 52 samples were studied in *Theileria annulata* genome and 6 different PCR-RFLP patterns were observed. Due to the high sensitivity and specialty of PCR, this method can be used to check the carriers in large herds to eradicate the disease. In the case of having educated and sophisticated human resource, this method can be used in high-inflected regions instead of blood smear. However, sporadic cases are not affordable due to high cost.

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