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DETECTION OF THEILERIA SPECIES AMONG IMPORTED AND LOCAL BREED FEEDLOT BULLS IN DUHOK GOVERNORATE–KURDISTAN REGION /IRAQ

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ABSTRACT

Background: **Bovine theileriosis is a tick-born disease in tropical and subtropical regions of the world. The study was aimed to detect *Theileria* sp. at genus level in blood samples of both local and imported feedlot bulls by direct Giemsa staining, ELISA, and nested polymerase chain reaction.**

Methods: A total of 250 blood samples (125 local and 125 imported feedlot bulls) were collected randomly. The samples were collected from five different districts of the Duhok Governorate in the Kurdistan region, Iraq from 1st July to 31st of October 2021. Giemsa stain and ELISA techniques were performed for all cases, while the nPCR was done for seropositive cases recognized by ELISA.

Results: The infection with *Theileria* parasite was 8.8% (n=22) by Giemsa staining and 11.2% (n=28) was seropositive by ELISA. The current study showed that 75% (n=21) of 28 seropositive cases by ELISA were positive by a nPCR technique. *Theileria* parasite was highest among imported breeds compared to the local breeds. The highest rate of *Theileria* parasite was in the Shekhan and the Summel districts of both imported and local breeds.

Conclusions: This study showed that the imported breeds have a higher rate of *Theileria* parasite but the overall difference was not statistically different (P=value 0.0772)

KEYWORDs: *Theileria* infection, bulls, Giemsa, ELISA, PCR

INTRODUCTION

Bovine theileriosis is a tick-borne hemoprotozoan disease of cattle caused by several *Theileria* species. At least five species of *Theileria* (*T. parva*, *T. annulata*, *T. taurotragi*, *T. velifera*, and members of *T. sergenti/orientalis*/buffeli group) have been found to infect the cattle. The most important species responsible for the disease are *T. annulata* and *T. parva* (Dantas- Torres and Otranto, 2016).

It is one of the most economically devastating diseases of livestock in North Iraq, mainly in Duhok Governorate and in most parts of the world. The disease-causing agent is transmitted by hard ticks and has a complicated life cycle (Morrison, 2015). Prominent signs that have been exhibited by the infected bull are anorexia, fever, enlarged lymph nodes, occulonasal discharges, and diarrhea (Kundave *et al*., 2015).

The clinical signs and microscopic examination are usually dependable for the diagnosis of piroplasms infection since it is easy and less expensive. However, the lack of adequate sensitivity and accuracy of the staining method may lead to a false diagnosis. Although serological tests are being used for the detection of latent infection, chances of false positive and negative results are likely to occur (Mehlhorn, 2001b; Saeid *et al*. 2013). The PCR method is more accurate in comparison with the ELISA, as well as the microscopic detection of piroplasms forms (Gubbles *et al*. 2000).

Tropical theileriosis affects cattle and water buffaloes in tropical and northern subtropical regions of the old world. The disease occurs when there is much tick activity, mainly during summer (Dumanl and Özer, 1987; Pipano, 1991; Keleş *et al*., 2001) but a single tick can cause fatal infection (Radostits *et al*., 2000).

In temperate and tropical regions where they represent serious issues for the management and health of cattle, tick-borne protozoan illnesses (such as theileriosis and babesiosis) are to blame for annual losses of billions of dollars (Jongejan and Uilenberg, 1994; Jongejan and Uilenberg, 2004; Ahmed *et al*., 2008).

Without vaccination or strict tick population management, all susceptible cattle in endemic locations run the danger of getting tropical theileriosis (Radostits *et al*., 2000). Case fatality

varies considerably from 5% or less in indigenous breeds of cattle to 20 - 90% in exotic cattle breeds depending on *T. annulata* strain pathogenicity and the degree of the challenge, or the number of parasites spread by all the ticks (Neitz, 1957; Solusby, 1982; Levine, 1985; Radostits *et al*., 2000; Mehlhorn, 2001b).

The virulence of *Theileria* species strains and the dose of infection affect the course of theileriosis as a disease generally (Mehlhorn, 2001b). Large doses of sporozoites of *T. annulata* may cause acute lethal disease in susceptible animals, especially in foreign breeds imported to a disease-endemic area (Preston *et al*., 1992). The innate resistance or susceptibility of the animal population, as well as variations in cow breeds, are other factors that affect the course of the disease (Bakheit and Latif, 2002).

The parotid lymph node is considered one of the first clinical signs of east coast fever, then is followed by lymphadenopathy, there is rising in fever and it remains rising just before death, the high in temperature is fast and exceeds 42 degrees. There are petechial and ecchymotic hemorrhages on the membranes of the conjunctiva and the buccal cavity (Mehlhorn, 2001b). Anorexia progress follows by loss of body weight. (Solusby, 1982; Levine, 1985; Radostits *et al*., 2000).

Because of little information about theileriosis in bulls in the Duhok governorate, the present study is conducted and aimed to

1- Detect the *Theileria* parasite by three laboratory techniques (Direct Giemsa staining of blood film, ELISA, and nested PCR).

2- Determine the roles of importing feedlot bulls in the distribution of theileriosis among the local breeds in the Duhok province.

3- Compare techniques in the detection of the parasite.

MATERIALS AND METHODS

Study design, setting, and animal sampling

In the current cross-sectional study, a total of 250 feedlot bulls (125 local breeds and 125 imported breeds) were randomly collected. The cases were collected from five districts of the Duhok governorate. The districts were Duhok center, Summel, Amedi, Zakho, and Shekhan. The cases were collated from 1st July to 31st October 2021.

Bulls sampling

Two hundred and fifty feedlot bulls were randomly selected in which every 50 bulls were selected from each district. Of these 50 bulls, 25 cases were from the local and 25 from imported breeds. The ages of bulls were between one year and three years.

Collection of samples

A. Blood samples

A Ten-milliliter blood sample was withdrawn from the jugular vein of each animal by a 10 ml disposable syringe with an 18-gauged needle. The blood was emptied into two commercially prepared EDTA and sera Clute activator gel tubes. All tubes were labeled with specific numbers and kept in an insulated container with ice packs. The samples were submitted to the laboratory for preparation of sera, and blood smears. The remaining blood samples were stored at -20°C until used for the DNA extraction.

B. Blood smears preparation and Giemsa staining of smears

Blood smears of all sampled animals were prepared according to Coles (1986), briefly, a small drop of blood was put on a grease-free slide, the drop was spread by a smoothed-edge glass slide, and the smear was air-dried and labeled. The prepared smears were fixed in methyl alcohol for 5 minutes and air-dried, then immersed in working Giemsa staining solution for 30 minutes. Washed in distilled water and finally air-dried.

Microscopic examination of the Giemsa-stained smears

Giemsa-stained blood smears were examined using the oil-immersion lens (X100) of the light microscopy.

Processing of blood samples for ELISA

Serum preparation:

Sera were collected from blood in Clute activator gel tubes are processed as bellow:

Bovine anti *Theileria* IgG antibody detection by ELISA:

Assay principle:

This kit was based on a qualitative reverse-phase enzyme immunoassay technique. The microtiter plate has been pre-coated with a target antigen. Positive/negative controls or samples were added to the wells and incubated. Antibodies in the samples were bound to the antigen on the plate. Unbound antibody was washed away during a washing step. A Horseradish peroxidase (HRP) conjugated detection antibody was then added and incubated. Unbound HRP has washed away during a washing step. TMB (3,3',5,5'-Tetramethylbenzidine) is a substrate

for horseradish peroxidase (HRP) substrate was then added and color develops. The reaction was stopped by the addition of an acidic stop solution and the color was changed to yellow which can be measured at 450 nm. The OD of an unknown sample was compared to the OD of the positive and negative controls to determine the presence of anti *Theileria* IgG Antibody.

PCR assay

DNA extraction from blood samples

DNA from blood samples was extracted according to (BIO-KOREA) using the DNA extraction kit (KOREA)

PCR assay

The PCR was performed on 28 samples that were seropositive by the ELISA.

Specific primers for *Theileria* spp as shown in (Table 1) targeting 18S rRNA genes were used to amplify the P104 gene by using nested polymerase chain reaction (nPCRs) according to (Cao, *et al*, 2002). Initial PCR amplifications were done in a 20 µl-reaction mixture containing 2 µl of DNA template, 1 µl (10 µM) of each primer, 10µl of add taq master mix (Addbio/ Korea), and 6 µl of double distilled water. Nested PCR was done using a 20 µl-reaction mixture containing 1 µl (10 µM) of each primer, 10µl of Addtaq master mix (Addbio/ Korea), and 7 µl of double distilled water and1 µl of DNA template obtained from the first PCR amplification. Thermal cycling was performed based on (reference) in an automatic DNA thermal cycler (Eppendorf, Germany). Generally, the PCR set was started at 95 °C for 5 minutes as an initial denaturation followed by 35 cycles of 94 °C for 45 seconds, 55 °C for 50 seconds, and 72 °C for 1 minute. The final extension was at 72 °C for 7 minutes.

Table (1): primers used in nPCR protocol.

Pathogen /target gene	Primers	Oligonucleotide sequences (5' - 3')	Product Molecular size (bp)	Reference
Theileria spp./ 18S rRNA	Outer primer	GAAACGGCTACCACATCT AGTTTCCCCGTGTTGAGT	778	Cao *et al.* (2002)
	Inner primer	**TTAAACCTCTTCCAGAGT** **TCAGCCTTGCGACCATAC**	581	

Theileria DNA positive control (obtained from the Duhok research center, College of the Veterinary medicine/ university of Duhok) was used with each PCR reaction, whereas. The double distilled water was used as a negative control. The nPCR products were electrophoresed in 1 X TAE buffer at 85 volts for 45 min and then visualized under a UV transilluminator.

Statistical analyses

The rate of positive ratio of theileriosis by Gimsa stain, ELISA, and PCR of total, local, and imported breeds were determined in number and percentage. The difference in the rate of theileriosis by Giemsa stain, ELISA, and PCR among different locations were examined in a Pearson chi-squared test. The comparison of positive results of theileriosis of PCR, LISA, and Gimsa was examined in a Pearson chi-squared test. Sensitivity, specificity, and accuracy results of ELISA over Gimsa and PCR over Gimsa in the diagnosis of theileriosis in total, local, and imported breeds were calculated as follows:

True positive (TP): A test result that correctly indicates the presence of a condition or characteristic

True negative (TN): A test result that correctly indicates the absence of a condition or characteristic

False positive (FP): A test result that wrongly indicates that a particular condition or attribute is present

False negative (FN): A test result that wrongly indicates that a particular condition or attribute is absent

$$Sensitivity = \frac{TP}{TP + FN} * 100$$

$$Specificity = \frac{TN}{TN + FP} * 100$$

$$PPV\ (positive\ predicitve\ value) = \frac{TP}{TP + FP} * 100$$

$$NPV\ (negative\ predicitve\ value) = \frac{TN}{TN + FN} * 100$$

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN} * 100$$

RESULTS

The infection rate of theileriosis by Giemsa stain:

The total positive rate of infection, local breeds, and imported breeds were 8.8%, 4.8%, and 12.8%, respectively. The highest rate of theileriosis (20%) was observed in the Shekhan district among imported feedlot bulls, whereas the second highest rate of the infection (16%) was found in the Summel district among imported breed feedlot bulls. whereas the third highest rate (12%) of the infection was found in the Zakho district among local breed feedlot bulls and the no infection (0%) was found in the Duhok center among local breeds of feedlot bulls. The infection rates mentioned above showed that the rate of theileriosis in imported breeds to local breeds was (12.8% - 4.8%) and was approximately higher by (8%) than in local breed feedlot bulls. There was no statistically significant difference in the infection rate of theileriosis in bulls by Giemsa statin at P value 0.4202 (Table 2, Figure 1).

Table(2): Infection rate of theileriosis in bulls by Giemsa stain according to districts in Duhok Governorate.

	Giemsa stain									
Location	**Sample number**	**Local breeds**	**Imported breeds**	**Positive samples (total)**	**Positive (local breed sample)**	**Positive (imported breed sample)**	**Positive ratio (Total)**	**Positive ratio (Local breeds)**	**Positive ratio (Imported breeds)**	**P-value**
Duhok center	50	25	25	2	0	2	4.0	0.0	8.0	**0.4204**
Sumeel district	50	25	25	6	2	4	12.0	8.0	16.0	
Amedi district	50	25	25	3	1	2	6.0	4.0	8.0	
Zakho district	50	25	25	4	1	3	8.0	4.0	12.0	
Shekhan district	50	25	25	7	2	5	14.0	8.0	20.0	
Totals	250	125	125	22	6	16	8.8	4.8	12.8	

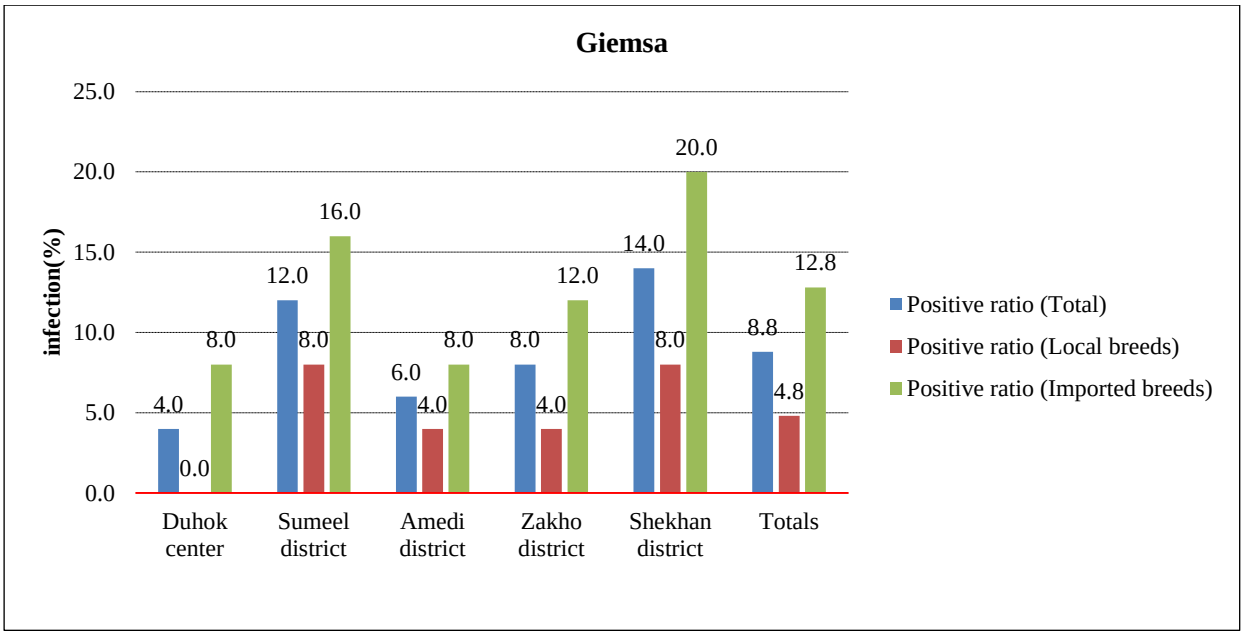
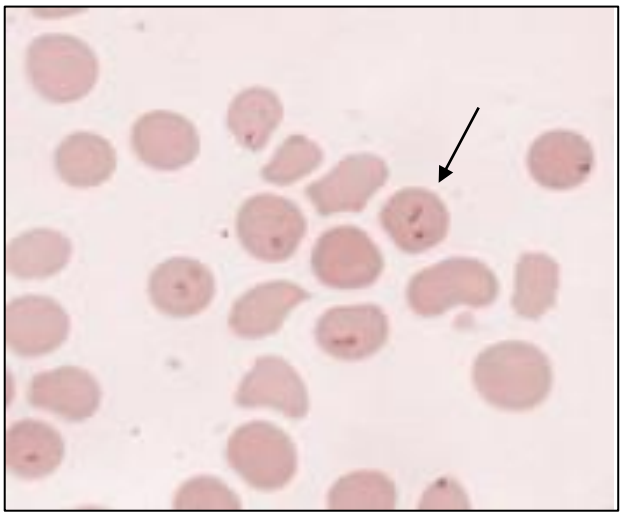


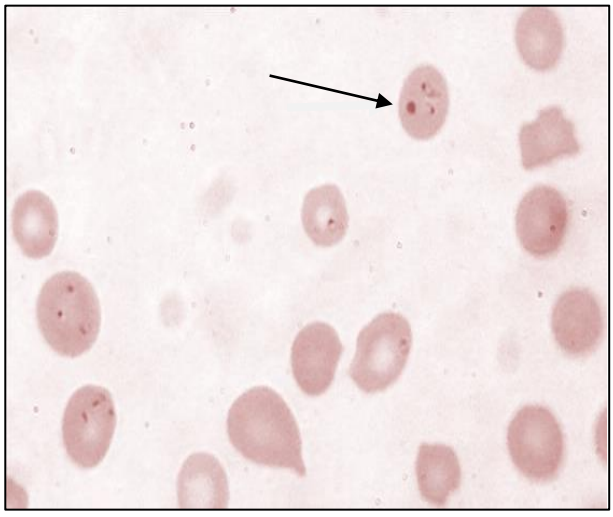
Fig.(1): Infection rate of theileriosis in bulls by Giemsa stain according to districts in Duhok Governorate.

***Theileria* species parasites in a blood smear stained by Giemsa-stain**

The microscopic examination of the blood smears stained by Giemsa-stain of infected bulls showed the presence of free and intracellular shapes morphologically well-matched with *Theileria* parasites and schizonts stages. Infected erythrocytes showed morphological disorders represented by round-shaped appearance and irregular thorn-like protrusions. The piroplasms within their cytoplasm were mainly round or oval but the rod and comma forms have also been identified. They were observed as an single parasite (one piroplasm per erythrocyte), double, triple, and tetra-shapes. The schizonts were observed either as free forms or as intracellular forms in some of the white blood cells like the monocytes and lymphocytes within the blood smears. They appeared as circular or irregularly shaped structures with blue cytoplasm and varied numbers of red chromatin granules (figure 2,A,B).

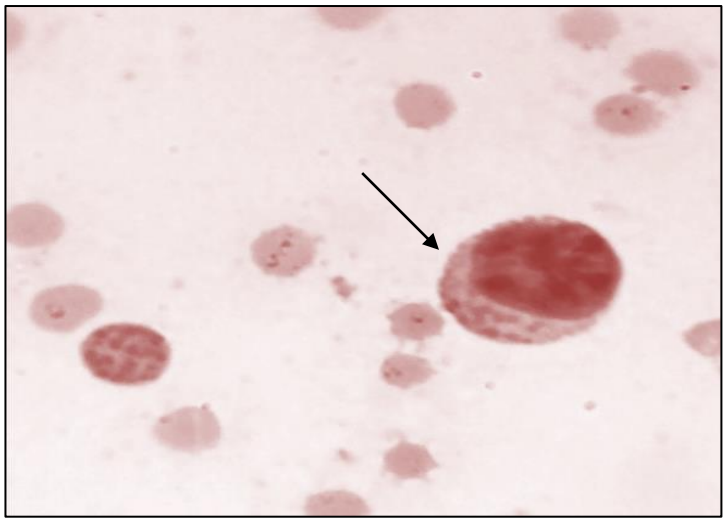


A



B

(Figure 2, C): Giemsa-stained blood smears of infected bull reveal the presence of intra-erythrocytic forms (arrows) morphologically compatible with *Theileria* stages. Some of the infected erythrocytes showed morphological disorders represented by round-shaped appearance and irregular thorn-like protrusions (X 100). Blood smears stained by Giemsa-stain of infected bull reveal the presence of extracellular (thick arrow) and intracellular (thin arrows) schizont forms of *Theileria* parasites. Intra-erythrocytic piroplasm forms are also evident (X 100).



C

The seropositivity of anti *Theileria* IgG Antibody by ELISA

The highest seropositivity (16%) of theileriosis was observed in all three districts by the same ratio (Duhok, Summel, and Shekhan districts) among imported feedlot bulls. Whereas, the second highest seropositivity (12%) was found in Amedi and Zakho districts among imported breed feedlot in the same ratio was found among local breeds of the Amedi district. On the other hand, the third highest seropositivity (8%) was found in the Duhok center among local breed feedlots bulls and the lowest seropositivity (4%) found in Zakho and Shekhan districts among local breeds of feedlot bulls. The seropositive cases mentioned above showed that the infection rate of theileriosis in imported breeds to local breeds was 14.4% - 8%) and was approximately highest 6.4% than in local breed feedlot bulls (Table 3).

Table (3): Seropositivity of anti *Theileria* IgG antibody in bulls by ELISA ac cording to districts**.**

ELISA

Location	Sample number	Local breeds	Imported breeds	Seropositive samples (total)	Seropositive (local breed sample)	Seropositive (imported breed sample)	SeropPositive ratio (Total)	Seropositive ratio (Local breeds)	Seropositive ratio (Imported breeds)	P-value
Duhok center	50	25	25	6	2	4	12.0	8.0	16.0	
Sumeel district	50	25	25	7	3	4	14.0	12.0	16.0	0.0772
Amedi district	50	25	25	6	3	3	12.0	12.0	12.0	
Zakho district	50	25	25	4	1	3	8.0	4.0	12.0	
Shekhan district	50	25	25	5	1	4	10.0	4.0	16.0	
Totals	250	125	125	28	10	18	11.2	8.0	14.4	

The infection rate of theileriosis in bulls by nested polymerase chain reaction test

The highest infection rate (100%) of theileriosis was observed in Sumeel and Amedi districts among imported feedlot bulls and the same rate (100%) was found among local breeds in the Zakho district,

whereas the second highest infection rate of the infection (75%) was found in the Duhok center among imported breed feedlot bulls. Whereas the third highest infection rate of 66.7% of the disease was found in the Zakho district among local breed feedlot bulls, the same rate of 66.7% was found among local breeds in the Summel district. The lowest infection rate (33%) was found in the Shekhan district among local breeds of feedlot bulls. The study results showed that the infection rate of theileriosis in imported breeds to local breeds was 77.78% - 50% and was approximately higher by 27.78% than in local breed feedlot bulls as shown in (Figure 3. 4) which illustrates the nested PCR result of *Theileria* in blood samples of bulls (local and imported breeds).

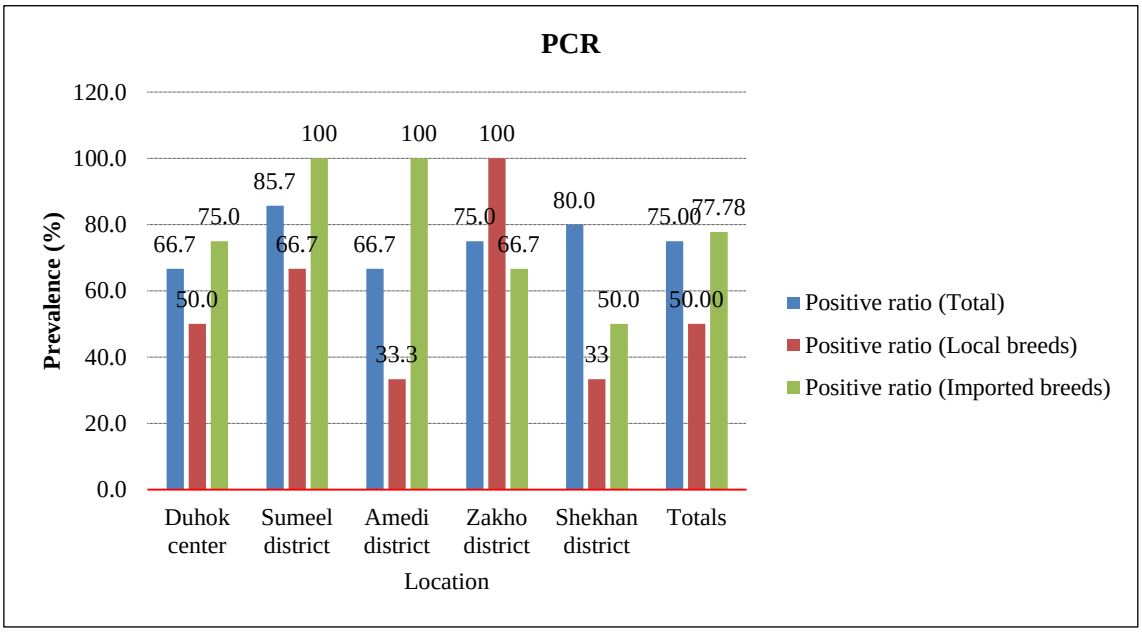


Fig. (3):- Infection rate of theileriosis in bulls by nPCR according to districts.

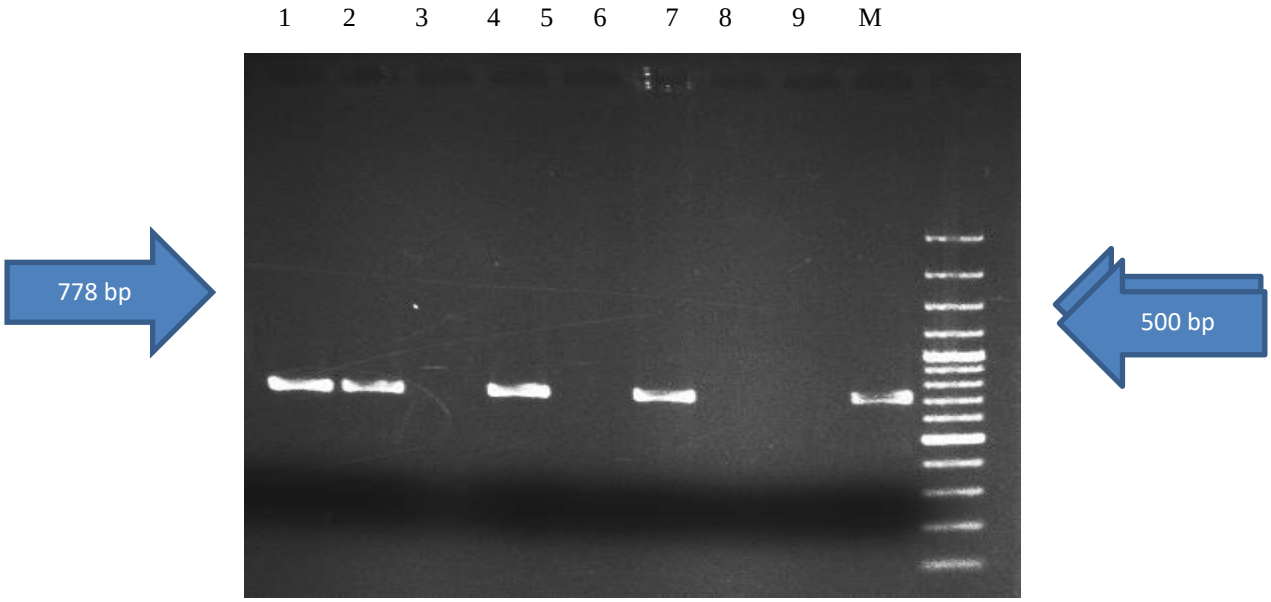


Fig.(4):- Agarose gel electrophoresis of nested PCR assay. Lanes 1, 2, 4, and 6 are positive samples with *Theileria*, whereas lanes 3, 5, and 7 are negatives. Lane 8 is control negative, lane 9 is control positive and (M) the marker.

Comparison of the results among three techniques

The positivity by the three techniques markedly differed revealing percent positivity of 12.8%–4.8% for both imported and local breeds feedlot bulls by Giemsa stain, 14.4% - 8% by ELISA and 77.78% - 50% by polymerase chain reaction test respectively (Table 4).

Out of the 250 stained blood samples tested microscopically, 22 were found positive and 228 were negative. While a total of samples 250 tested by the ELISA, 28 were found seropositive and 222 were negative, whereas the 28 seropositive samples by ELISA, only 21 of which were found positive by nPCR technique and 7 samples were negative.

The comparison of the positive results of the three techniques will be as follow: 8.8%, 11.2%, and 75% for Gram stain, ELISA, and nPCR, respectively. But 4.8%, 8%, and 50% for Giemsa stain, ELISA, and nPCR sequentially for local breed feedlot bulls. Whereas 12.8%, 14.4%, and 77.78 for Gram stain, ELISA, and nPCR sequentially for imported breed feedlot bulls. So it is clear that the percentage of positive samples among imported feedlot bulls is much higher than among local breed feedlot bulls (Table 4, figure 5, 6 and 7).

Table (4): Comparison of positive results of theileriosis of PCR, ELISA, and Giemsa

	Giemsa no (%)		**ELISA no (%)**		**PCR no (%)**		**p-value (two-sided)**
Location	**Number**	**Positive ratio (Total)**	**Number**	**Positive ratio (Total)**	**Number**	**Positive ratio (Total)**	
Duhok center	50	2 (4.0)	50	6 (12.0)	6	4 (66.7)	
Sumeel district	50	6 (12.0)	50	7 (14.0)	7	6 (85.7)	
Amedi district	50	3 (6.0)	50	6 (12.0)	6	4 (66.7)	0.6191
Zakho district	50	4 (8.0)	50	4 (8.0)	4	3 (75.0)	
Shekhan district	50	7 (14.0)	50	5 (10.0)	5	4 (80.0)	
Total	250	22 (8.8)	250	28 (11.2)	28	21 (75.0)	
	Giemsa no (%)		**ELISA no (%)**		**PCR no (%)**		
Location	**Local breeds**	**Positive ratio (Local breeds)**	**Local breeds**	**Positive ratio (Local breeds)**	**Local breeds**	**Positive ratio (Local breeds)**	
Duhok center	25	0 (0.0)	25	2 (8.0)	2	1 (50.0)	
Sumeel district	25	4 (8.0)	25	3 (12.0)	3	2 (66.7)	
Amedi district	25	2 (4.0)	25	3 (12.0)	3	1 (33.3)	0.2771
Zakho district	25	2 (4.0)	25	1 (4.0)	1	1 (100)	
Shekhan district	25	4 (8.0)	25	1 (4.0)	3	1 (33)	
Total	125	6 (4.8)	125	10 (8.0)	12	6 (50.0)	
	Giemsa no (%)		**ELISA no (%)**		**PCR no (%)**		
Location	**Imported breeds**	**Positive ratio (Imported breeds)**	**Imported breeds**	**Positive ratio (Imported breeds)**	**Imported breeds**	**Positive ratio (Imported breeds)**	
Duhok center	25	2 (8.0)	25	4 (16.0)	4	3 (75.0)	
Sumeel district	25	4 (16.0)	25	4 (16.0)	4	4 (100)	
Amedi district	25	2 (8.0)	25	3 (12.0)	3	3 (100)	0.4232
Zakho district	25	3 (12.0)	25	3 (12.0)	3	2 (66.7)	
Shekhan district	25	5 (20.0)	25	4 (16.0)	4	2 (50.0)	
Total	125	16 (12.8)	125	18 (14.4)	18	14 (77.78)	

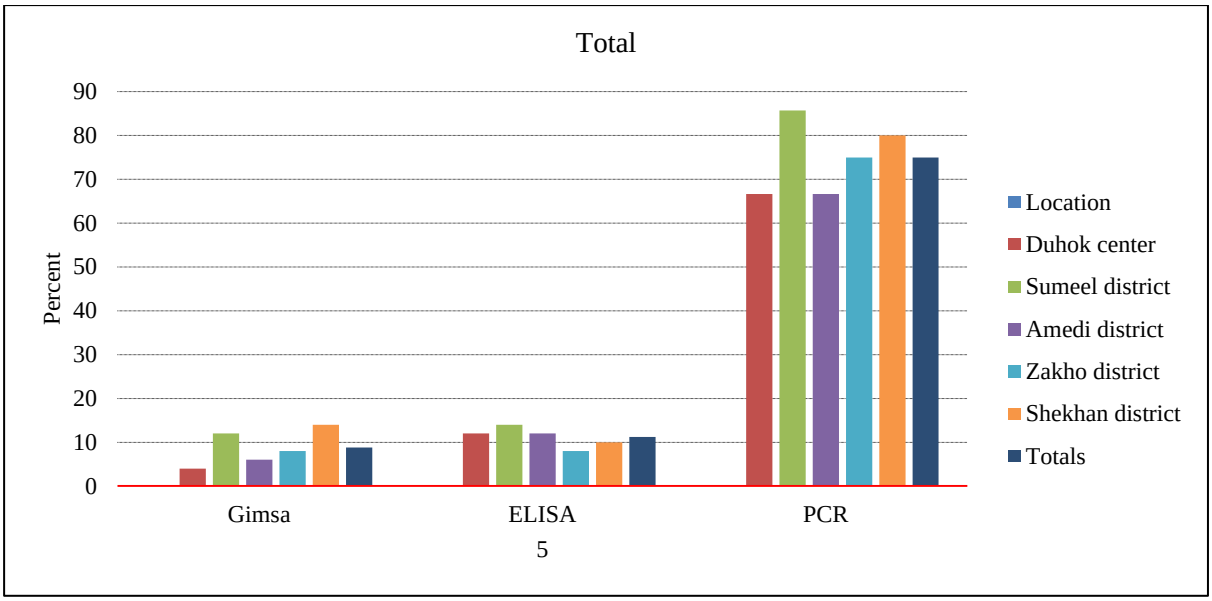


Fig.(5): Comparison of positive results of theileriosis of nPCR, ELISA, and Giemsa stain in general revealing percent positivity of (12.8% – 4.8%) for both imported and local breeds feedlot bulls by Giemsa stain, (14.4% - 8%)by ELISA and (77.78% - 50%) nPCR respectively.

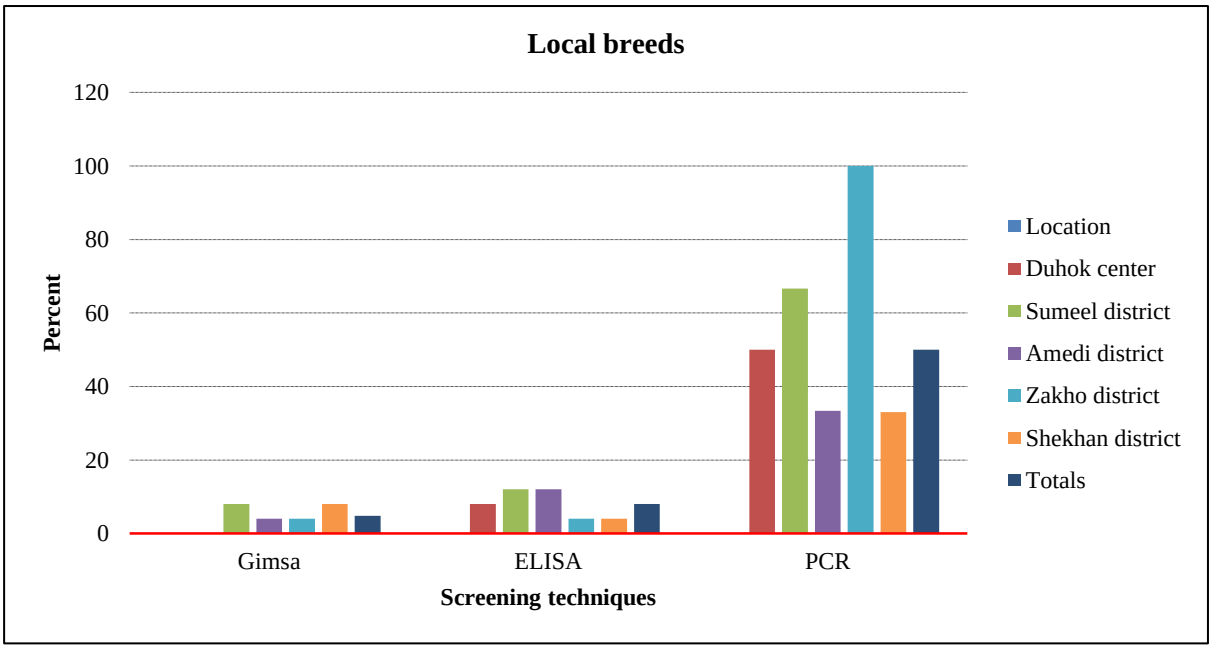


Fig.(6): Comparison of positive results of theileriosis of nPCR, ELISA, and Giemsa stain reveal (4.8%, 8%, and 50%) for Gram stain, ELISA, and nPCR sequentially for local breed feedlot bulls.

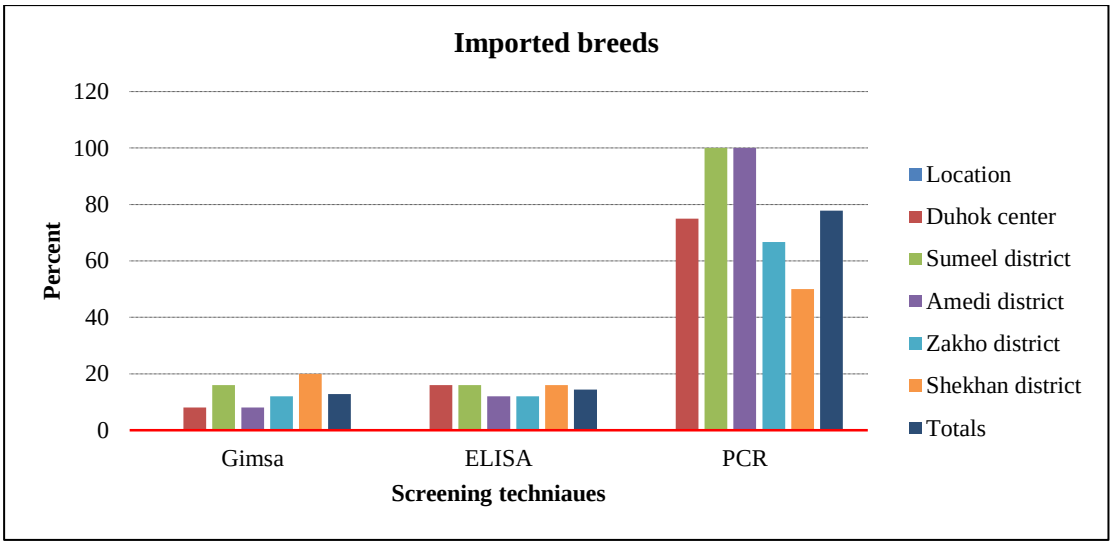


Fig.(7): Comparison of positive results of theileriosis of nPCR, ELISA, and Giemsa stain that reveal the percentage of positive samples among imported feedlot bulls.

Table (5): Sensitivity, specificity, and accuracy results of ELISA and Giemsa Stain in the diagnosis of theileriosis in total, local, and imported breeds

Totals	**Giemsa**		**ELISA**		**Accuracy results of total breeds**				
Location	**Number**	**Positive samples (total)**	**Number**	**Positive samples (total)**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
Duhok center	50	2	50	6	3.8	92.3	33.3	49.0	48.1
Sumeel district	50	6	50	7	10.7	97.8	85.7	46.8	49.5
Amedi district	50	3	50	6	5.7	94.0	50.0	48.5	48.5
Zakho district	50	4	50	4	7.4	100	100	47.9	50.0
Shekhan district	50	7	50	5	12.3	95.6	77.8	46.2	49.0
Totals	250	22	250	28	8.1	97.4	78.6	47.7	49.4
Local breeds	**Giemsa**		**ELISA**		**Accuracy results of local breeds**				
Location	**Local breeds**	**Positive (local breed sample)**	**Local breeds**	**Positive (local breed sample)**					
					Sensitivity	**Specificity**	**PPV**	**NPV**	**Accuracy**
Duhok center	25	0	25	2	0.0	92.6	0.0	50.0	48.1
Sumeel district	25	2	25	3	7.4	95.8	66.7	47.9	49.0
Amedi district	25	1	25	3	3.8	92.3	33.3	49.0	48.1
Zakho district	25	1	25	1	3.8	100	100	49.0	50.0
Shekhan district	25	2	25	1	7.4	95.8	66.7	47.9	49.0
Totals	125	6	125	10	4.6	96.7	60.0	48.8	49.2
Imported	**Giemsa**		**ELISA**		**Accuracy results of imported breeds**				
Location	Imported breeds	Positive (imported breed sample)	Imported breeds	Positive (imported breed sample)					
					Sensitivity	Specificity	PPV	NPV	Accuracy
Duhok center	25	2	25	4	7.4	92.0	50.0	47.9	48.1
Sumeel district	25	4	25	4	13.8	100	100	45.7	50.0
Amedi district	25	2	25	3	7.4	95.8	66.7	47.9	49.0
Zakho district	25	3	25	3	10.7	100	100	46.8	50.0
Shekhan district	25	5	25	4	16.7	95.2	83.3	44.4	49.0
Totals	125	16	125	18	11.3	98.2	88.9	46.6	49.6

The Sensitivity, specificity, and accuracy results of ELISA and Giemsa stain in the diagnosis of theileriosis in total are (8.1, 97.4, and 49.4) as shown in (Table 5 and 6) sequentially. There was a fair agreement between microscopy and ELISA results. And low sensitivity of light microscopy of Giemsa-stained blood smears is attributed to the fact that this method is dependable for the detection of acute cases but has limited value for chronic and long-lasting carrier cases, where only low numbers of *Theileria* stages exist.

The sensitivity, specificity, and accuracy results of nPCR and Giemsa stain in the diagnosis of theileriosis in total are (9.6, 99.6, and 54.7) as shown in (Table 6) sequentially. There was a fair agreement between microscopy and nPCR results. And low sensitivity of light microscopy of Giemsa-stained blood smears is attributed to the fact that this method is dependable for the detection of acute cases but unlike nPCR has limited value for chronic and long-lasting carrier cases, where only low numbers of *Theileria* stages exist.

Table (6): Sensitivity, specificity, and accuracy results of nPCR and Giemsa stain in the diagnosis of theileriosis in total, local, and imported breeds

Totals	**Giemsa**		**PCR**		**Accuracy results of total breeds**				
Location	**Number**	**Positive samples (total)**	**Number**	**Positive samples (total)**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
Duhok center	50	2	6	4	4.2	96.0	50.0	51.1	51.0
Sumeel district	50	6	7	6	13.6	100	100	53.7	56.8
Amedi district	50	3	6	4	6.4	97.9	75.0	51.6	52.6
Zakho district	50	4	4	3	8.7	97.9	80.0	52.3	53.8
Shekhan district	50	7	5	4	16.3	93.5	70.0	54.4	56.2
Total	250	22	28	21	9.6	99.6	95.7	52.5	54.7
Local	**Giemsa**		**PCR**		**Accuracy results of local breeds**				
Location	**Local breeds**	**Positive (local breed sample)**	**Local breeds**	**Positive (local breed sample)**					
					Sensitivity	**Specificity**	**PPV**	**NPV**	**Accuracy**
Duhok center	25	0	2	1	0.0	96.2	0.0	50.0	49.0
Sumeel district	25	2	3	2	8.7	100	100	52.3	54.3
Amedi district	25	1	3	1	4.2	100	100	51.1	52.1
Zakho district	25	1	1	1	4.2	100	100	51.1	52.1
Shekhan district	25	2	2	1	8.7	95.8	66.7	52.3	53.2
Total	125	6	11	6	5.0	100	100	51.3	52.5
Imported	**Giemsa**		**PCR**		**Accuracy results of imported breeds**				
Location	**Imported breeds**	**Positive (imported breed sample)**	**Imported breeds**	**Positive (imported breed sample)**					
					Sensitivity	**Specificity**	**PPV**	**NPV**	**Accuracy**
Duhok center	25	2	4	3	8.7	95.8	66.7	52.3	53.2
Sumeel district	25	4	4	4	19.0	100	100	55.3	59.5
Amedi district	25	2	3	3	8.7	95.8	66.7	52.3	53.2
Zakho district	25	3	3	2	13.6	95.7	75.0	53.7	55.6
Shekhan district	25	5	4	2	25.0	87.0	62.5	57.1	58.1
Total	125	16	18	14	14.7	98.2	88.9	54.0	56.8

DISCUSSION

The overall infection rate of theileriosis in the populations of both types of bulls studied in the current investigation revealed that the rate in imported feedlot bulls was around 2.1 times greater than that from local breed feedlot bulls. This discovery is directly attributed to vector activity in different regions around the two different groups of bulls. The disease manifests itself when there is a high tick population in the surrounding animal environment (Dumanl and zer, 1987; Pipano, 1991; Keleş *et al.,* 2001).

In the Duhok districts, the rate values of theileriosis found in bulls in a few different geographic areas showed that the rate found in imported breed feedlot bulls is twice times higher than the rate found in local breed feedlot bulls in almost all five regions of the study within Duhok districts. Several epidemiological factors, such as the various herd management systems, disease prevention measures, the age of animals sampled, and the various weather conditions in various districts of the Duhok region, may be responsible for this variation in rate values of theileriosis. The final and maybe

most significant component is connected to the biological activity of the vector tick, which is affected by the ambient temperature since cold weather significantly prolongs the various developing phases of ticks (Solusby, 1982). According to research by Bakheit and Latif, Glass *et al*., 2005, Ahmed *et al*., 2008, and other authors (Sayin *et al*., 2003; Dumanl *et al*., 2005; Aktas *et al*., 2006; Inci *et al*., 2008), crossbreed and imported bulls are more susceptible to theileriosis than native or local breed bulls. This may account for the seeming higher prevalence of the disease in imported bulls.

Regarding the age of bulls that were used in the present study, a clear increase in the rate of theileriosis was noted in animals older than 2 years compared to those of less than two years of age. Once again, the likelihood that young bulls are largely kept indoors and so at a lower risk of tick infestation is the cause of this age-related frequency difference. This outcome is largely consistent with research findings from earlier studies they noted that the incidence of theileriosis rises as age increases (Flach and Ouhelli, 1992; Flach *et al*., 1995; Darghouth *et al*., 1996b; Darghouth *et al*., 1999).

The results obtained in the current study are the same as the findings of Hoghooghi *et al*. 2011 who showed the prevalence of theileriosis to be 6.25 % (10 positive cases out of 160 samples) by the staining method. The prevalence of *Theileria* infection by PCR assay was found to be 70 % (21 out of 30) while by Giemsa staining method was 30 % (9 out of 30) in a study conducted by Mahmmod *et al*. 2010. Roy *et al.* (2000) reported that the number of positive cases of theileriosis out of 50 blood samples of local breed bulls by PCR and stained smear method were 22 (44 %) and 8 (16 %), respectively. Saeid *et al*. 2013 found that 68 of 150 (45.33 %) of the carrier bulls were positive by PCR. Twenty-one samples were exceedingly positive by PCR indicating its higher sensitivity over microscopy and ELISA. Seven negatives by PCR could be detected as seropositive by ELISA which indicated the persistence of antibodies after clearance of infection. therefore

The results obtained in the present study indicate that nPCR is more accurate in the detection of theileriosis than the conventional staining technique and ELISA and is in agreement with previous studies (Roy *et al*. 2000; Mahmmod *et al*. 2010; Hooghooghi *et al*. 2011) and (Saeid *et al*. 2013).

Nested PCR and sequencing were used to determine the prevalence of the *Theileria* parasites in 28 blood samples collected from local and imported breeds of feedlot bulls. *Theileria* spp. 18S rRNA, *T. parva* sp104, and *T. orientalis* major piroplasm surface protein were used as the marker genes, and the PCR assay was found to be sensitive and specific. Ananda *et al*. 2009 recorded a higher prevalence of *Theileria* in aged bulls, which is in agreement with the present study in which the highest prevalence was recorded in aged and imported bulls.

In the present study, the Giemsa-stained blood smears had shown false negatives in visual examination under a light microscope, which shows the low sensitivity of this test. It may be due to several reasons made during the examination of slides, very low parasitemia, destruction of piroiplasmic forms in red blood cells due to hemolysis, the thickness, dirtiness, or unsuitable blood smear staining (Hoghooghi *et al*. 2011). Moreover, the microscopic detection of piroplasms in samples that were negative by PCR tests was not possible. This fact, confirms the superiority of PCR over blood smear examination. Statistical comparison of blood smear and PCR had shown a significant difference at a 5 % level.

Morphology of *T. annulata* parasites in Giemsa-stained blood smears

Bull erythrocytes infected with the piroplasm forms of the parasite *Theileria* had a round appearance and irregular thorn-like protrusions, according to Giemsa-stained blood smears. The following are the erythrocytic morphologic disorders: that are attributed to the erythrocyte oxidation, immune-mediated processes, and parasite presence in the erythrocytes (Yagi *et al*., 1989; Stockham *et al*., 2000; Singh *et al*., 2001a; Yagi *et al*., 2002), and they are generally in agreement with (Hoghooghi *et al*. 2011).

The identification of intracellular (in some of the monocytes and lymphocytes) and extracellular schizont forms of *Theileria* parasites in these Giemsa-stained blood smears was an intriguing discovery. The existence of *Theileria* schizonts outside of host cells has not yet been explained precisely, but it is possible to speculate that this finding is explained by an apoptosis-related mechanism "as it has been described for the malaria parasite (Sturm *et al*. 2006)" by which the parasites induce host cell apoptosis, causing the parasite to be released from the host cell and localized extracellularly.

The majority of the schizont forms of *Theileria* spp. (China) of sheep and goats are found outside the host cells, as noted by (Yin *et al*. 2007), who also found this conclusion to be generally consistent.

Sensitivity of the ELISA assay compared with Giemsa–stained blood smear

ELISA is considered an excellent tool for detecting *Theileria* antibodies, especially in chronic and carrier animals. During the acute phase of the disease, it is recommended to use another assay since the ELISA is unlikely to detect antibodies during the first week of infection (AL-Husary, 2013). Results of the current investigation confirmed that the thin blood smear is a specific but moderately sensitive diagnostic test. Although it is a cheap, easy, and fast test, it is limited to detection of the acute infection, when the level of parasitemia is high enough to be detected microscopically. These findings are in agreement with previous results obtained (AL-Husary, 2013).

Sensitivity of the PCR assay compared with Giemsa–stained blood smear

Considering blood smear examination as the gold standard assay, the sensitivity of the PCR method was found to be excellent (Sanchez *et al*. 1999).

The present study findings are in agreement with (Azizi *et al*. 2008) who reported that the sensitivity and accuracy of PCR in the detection of *Theileria* spp. superior to the blood smear examination. (Sanchez *et al*. 1999) verified the utility of this technique in epidemiological studies and compared it with conventional diagnostic techniques and reported that PCR was more sensitive for the detection of *Theileria* infections.

Dropping in the parasitemia occurs through drug therapy and thus delays accurate diagnosis (Ahmed and Mehlhorn 1999; Glass 2001). In such cases, PCR assays can be used to detect low parasitaemias in the blood of carrier bulls. The difficulties faced in the detection and differentiation of *Theileria* stages by conventional method (Giemsa stain and ELISA technique) is overcome by molecular methods like PCR. The high efficacy and sensitivity of PCR make it an attractive tool for diagnosing tick-borne infections (Olivier *et al*.1999). Hence, our study proves that PCR can be used for accurate diagnosis of theileriosis, and can also be used to detect the carrier animals, which serve as a potential source of infection to the healthier groups through the infected ticks.

In conclusion, theileriosis represents a major problem in the bull breeding in the Duhok region, in which the infection rate of the disease is higher in the warm districts of the Duhok region than in the cool districts. A high incidence of infection can be seen in aged bulls than in small ones. Imported animals have a great role in distributing the problem in our region. Finally, this investigation has proved that the PCR technique has superior for *Theileria* detection than other conventional tools (ELISA and direct Giemsa-stained blood). Therefore, according to the above-mentioned criteria, this study will recommend to putting a good restriction strategy especially on the country border to prevent the importing of animals from sources suspected to have *Theileria*.

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