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Research Article

MOLECULAR DETECTION OF PIROPLASMS IN CATTLE AT AL-NAJAF PROVINCE, IRAQ

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ABSTRACT

Background: *Theileria & Babesia* species are tick-borne haemoprotozoan parasites of vertebrates that had a chief force on domestic animals production, mainly cattle and small ruminants in tropical and subtropical areas. The diseases are still closely related to huge economic losses particularly in developing countries and Iraq; therefore, the aim of study is to determine tick-borne haemoprotozoan parasites infection in cattle of various ages based on ME and genetic methods from different regions in Al-najaf province of Iraq.

Materials & methods: A total of 150 blood samples were collected from February 2015 to February in 2016. Prevalence of infection was determined by Stained blood smears were microscopically examined for the presence of *Theileria* and *Babesia* organisms, and PCR was used for subsequent molecular specification hypervariable V4 region of the 18S ribosomal RNA (rRNA) gene was amplified with a set of primers for members of the genera *Theileria* and *Babesia*.

Results: PCR identified infection in 69.3 % (104/150) of the samples. There was a significant difference ($P \leq 0.05$) in the infection rate according to the tests.The Prevalence identified in cattle *Theileria* prevalence was 61.9% (78/126), and prevalence of *Babesia* was 20.6% (26/126) & the last one was less frequently detected. The results also suggest that the demographical picture of samples according to location & age, the Present results showed that the highest ratio in Al- meshkab (south of Al-najaf) was (27.8%). While the results showed that the age group between 2 to 3 years was the most vulnerable age group to piroplasms was recorded 58(46%), followed by age group (1-2) years 42 (33.3%).

Conclusions: the cattle in Al-najaf province were bearing the infection of piroplasmosis and recommended that the epidemiological condition calling for using more than two tests like microscopic examinations & PCR or ME & serological methods together.

INTRODUCTION

Theileria & Babesia species are tick-borne haemoprotozoan parasites of vertebrates that have a chief force on domestic animals production, mainly cattle and small ruminants in tropical and subtropical areas[1]. Also the diseases are still closely related to huge economic losses particularly in developing countries and Iraq[2]. Bovine *Babesiosis* (colloquially called Texas cattle fever, red water or piroplasmosis) was caused by infection with intraerythrocytic parasites, that were transmitted by bites from infected larval ticks of the order Ixodida *(Boophilus spp.)* and by the transovarial route[3]. *Theileriosis* caused by *Theileria* parasites infect a vast number of wild and domestic animals and are transmitted by various members of tick vectors of the family Ixodidae, (genus *Hyalomma*) [4].

Diagnosis was based on stained with Giemsa and the demonstration of parasite stages in blood or organ smears but this method was only suitable for the detection of acute cases and limited value for the detection of chronic because of the degree of parasitaemia in those animals[5]. Misdiagnosed with *piroplasma* in blood smears were significant similarity to the malaria parasite, *Plasmodium falciparum*[6] and not easy, not possible to discriminate pathogenic from non-pathogenic species that may occur simultaneously within the same host[7]. Serological methods were useful in diagnosing subclinical infections in epidemiological studies, but less accurate because of cross-reactivity with antibodies directed against other species of piroplasms[8] and also antibodies tend to disappear in long-term carriers, whereas piroplasms persist therefore, animals with a negative serological test can still be the source of the infection and infect ticks)[9]. PCR-based techniques provide an alternative method for the direct detection of

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piroplasms in carrier animals that a sensitive and highly specific method for the diagnosis[10]. In Iraq, *Theileriosis & Babesiosis* were one of the major factors preventing improvement of the productivity of livestock with reduce the production and growth of the diseased animals. However, little is known about the epidemiology and socio-economic impact of piroplasmosis in this region which were regarded as the problems associated with management and financial shortages [11,12,13]. Therefore, in present study, we focused on estimated the prevalence of piroplasmosis (Babesiosis and Theileriosis) among cattle in Al- najaf province\Iraq and compared the results of PCR to those of examination of thin blood smears, and then, previous aims lead to establish the good control measures and avoid unnecessary treatments in future.

MATERIALS AND METHODS

Collection of blood samples: the study was conducted on cattle in the Al-najaf province, Iraq from February to February in 2016, depend on questionnaire form was containing (location, species, gender, age, present or absence of tick), obtain information date from owner. A total of 150 blood samples were collected from the jugular vein of the animals into plain vacationer tube for laboratory examination.

Microscopic examination: in the laboratory, the blood smears were fixed in methyl alcohol for 15 min and stained for 30 min by Giemsa stain with 10%, examined for intra-erythrocytic forms of piroplasms at 100X objective magnification. Approximately 20000 erythrocytes per slide were examined for the calculation of percentage of infected erythrocytes. The smears were recorded as negative for piroplasms if no parasites were detected in oil-immersion fields[14].

Molecular detection: DNA Extraction: genomic DNA from blood samples were extracted by using Genomic DNA mini kit extraction kit (Frozen Blood) Geneaid. USA, and done according to company instructions.

Genomic DNA Profile: the extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm).

PCR Amplification: PCR technique was performed for detection and differentiation of *Theilera & Babesia spp.* based the digestion of 18S rRNA gene the method was carried out according to method described by[15].

Primers: 18S rRNA primers which used in PCR technique that used in detection and differentiation of *Theilera* spp and *Babesia* spp. This primers were designed by[15,6] and provided from Bioneer company, Korea as following table 1:

Table 1 Primers design

Primer		Sequence	PCR Amplicon
Theilera spp.	P1	CACAGGGAGGTAGTGACAAG	430bp
Babesia spp.	P2	AAGAATTTCACCTATGACAG	402bp

PCR master mix preparation*:* PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions as in table 2:

Table 2 Composition of PCR master mix

PCR Master mix	Volume
DNA template	5µl
18S rRNA P1 primer (10pmol)	1.5µl
18S rRNA P2 primer (10pmol)	1.5µl
PCR water	12µl
Total volume	20µl

After that, these PCR master mix component that mentioned above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes in PCR thermocycler, the conditions were done by using convential PCR thermocycler system as table 3:

Table 3 PCR conditions used in the study

PCR step	Tempreture	Time	repeat
Initial Denaturation	95°C	5min	1
Denaturation	95°C	30 Sec.	30 cycle
Annealing	58°C	30 Sec	
Extension	72°C	45 Sec	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

PCR product analysis: the PCR products of 18S rRNA gene were analyzed by agarose gel electrophoresis.

Statistical analysis: Chi-squared test was used to evaluate the differences among various parameters. P value less than 0.05 was accepted to be statistically significant.

RESULT

The present study indicated that diagnosis of *piroplasma* infections in (150) suspected cattle from different locations in Al-najaf city of the south-west of Iraq. *Theileria & Babesia* infection was microscopically detected in 126/150 (84%) of blood smears.

The demographical picture according to:

Location of studied samples was showed highest number of positive samples were obtained from Al-meshkab (south of Al-Najaf) with 35/126 (27.8%) that showed a high significantly rate (P value ≤ 0.005) than other regions which were presented in table 4.

Age groups, the present results showed that the age group between 2 to 3 years was the most vulnerable age group to piroplasms was recorded 58/126(46%), followed by age group (1-2) years 42/126 (33.3%) as in table 5.

Thin blood smears revealed piroplasms, detected inside erythrocytes, were pleomorph, ring or pear-shaped by using Gemsia´s stain. Of the 150 blood samples examined, microscopy revealed 126/150 (84 %) positive for piroplasms, whereas 104/150 (69.3 %) of PCR [78/126 (61.9%) and 26/126 (21.6%) for *Theileria & Babesia* respectively]. These results demonstrated that PCR showed a significant differences from did microscopic examination at the.05 level (P value ≤ 0.05) which are presented in table 6.

Table 4 The prevalence of piroplasms in cattle by microscopic examination from different reign in AL- Najaf city\Iraq

Region / Results		Al-meshkab	Al-Issa	Al-haidaria	Al- abbasid	Al- manathera	kufa	Total
Positive	Count	35	21	17	23	17	11	126
	Expected Count	31.9	17.6	14.3	23.5	14.3	16.8	126.0
Negative	Count	<5	<5	<5	5	<5	9	24
	Expected Count	6.1	3.4	2.7	4.5	2.7	3.2	24.0
Total	Count	38	21	26	17	17	20	150
	Expected Count	38.0	21.0	26.0	17.0	17.0	20.0	150.0

Pearson Chi-Square = 23.990 ; expected count = 2.72 ; df = 5 ; P ≤ 0. 005

Table 5 The incidence of piroplasms in cattle according to age

Age		frequencies				Total
		9.00	17.00	42.00	58.00	
1-2	Count	0	0	42	0	42
	% within frequencies	0.0%	0.0%	100.0%	0.0%	33.3%
2-3	Count	0	0	0	58	58
	% within frequencies	0.0%	0.0%	0.0%	100.0%	46.0%
3-4	Count	0	17	0	0	17
	% within frequencies	0.0%	100.0%	0.0%	0.0%	13.5%
≤ 4	Count	9	0	0	0	9
	% within frequencies	100.0%	0.0%	0.0%	0.0%	7.1%
Total	Count	9	17	42	58	126
	% within frequencies	100.0%	100.0%	100.0%	100.0%	100.0%

Pearson Chi-Square = 378.000, expected count = 0 .64 , P ≤ 0.0001

Table 6 The prevalence of *Theileria & Babesia* in cattle by microscopic examination & PCR*

Test		Result		Total
		Positive	Negative	
Gemsia	Count	126_a	24_a	150
	% within result	54.8%	52.2%	54.3%
PCR	Count	104_a	22_a	126
	% within result	45.2%	47.8%	45.7%
Total	Count	230	46	276
	% within result	100.0%	100.0%	100.0%

Pearson Chi-Square = 0.105 , expected count = 21.00 , P ≤ 0. 05
PCR*= Poly Chain Reaction

Table 7 The prevalence of *Theileria & Babesia* in cattle by PCR

Samples		frequencies			Total
		22.00	26.00	78.00	
Theileria	Count	0	0	78	78
	% within frequencies	0.0%	0.0%	100.0%	61.9%
Babesia	Count	0	26	0	26
	% within frequencies	0.0%	100.0%	0.0%	20.6%
Non found	Count	22	0	0	22
	% within frequencies	100.0%	0.0%	0.0%	17.5%
Total	Count	22	26	78	126
	% within frequencies	100.0%	100.0%	100.0%	100.0%

Pearson Chi-Square= 252.000 ; df = 4 ; expected count = 3.84 ; P ≤ 0.0001

DNA was extracted from the blood and amplified with primers derived from 18S rRNA gene of *Theileria* spp. and *Babesia* spp. The PCR products of *Theilera* spp. and *Babesia* spp. were 426-430 bp and 389-402bp, respectively. The PCR analysis showed an expected PCR product of (430, 4020)bp in length in DNA prepared from blood as in figure 1.

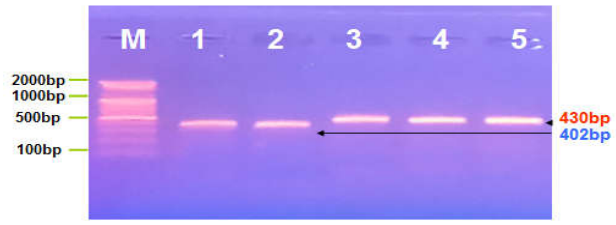

Figure 1 Agarose gel electrophoresis image that show the PCR product analysis of 18S rRNA gene that detection from cattle genomic DNA samples. Where M: Marker (2000-100bp), lane(1-2) positive PCR samples (*Babesia* spp.) at 402bp product size. Lane (3-5) positive PCR samples (*Theilera* spp.) at 430bp product size.

DISCUSSION

Ittle known about piroplasmosis in Iraq particularly in cattle and scarce data regarding the prevalence and occurrence of such diseases in south and middle region of Iraq. Accordingly, the objectives of this study were to determine the infectious rate of piroplasmosis (*Theileria & Babesia Spp*) in Al-najaf province. Microscopy using Giemsa stained blood smears had been considered the "gold standard" for detecting organisms in the blood of infected animals [16], and was applied to study the prevalence of infection from different regions. We have diagnosed 126 among 150 sampled of cattle (84%) from six regions (table 4). The most prevalent blood parasites in the regions were Al-meshkab (in the south of Al-najaf) which finding higher ratio (27.8 %) compared with other regions, that may be belonged to the types of the tick vectors were found in that region especially this region regarded as the first line in Agriculture of rice . However, ME was not sensitive enough or sufficiently specific to detect chronic or particularly when mixed infections occur, consequently used the molecular techniques based on PCR amplification and was performed for detection of bovine *Theileria and Babesia* using primers derived from 18S rRNA gene, our results recorded (69.3%) samples examined showed a positive signal (table 7), there's a significant differences between tests. In spite of the conventional PCR method was an effective and practical tool,

able to detect extremely low parasitaemia levels[17]. These results were in corresponding with the findings obtained by [18,19,20] who confirmed that the strong evidence of PCR was much more sensitive than ME either in clinically infected or apparently healthy ones (carriers).

The prevalence of *Babesia* infection (20.6 %) was lower than that of *Theileria* infection (61.9%). This result was agreed with[21] who revealed that 19%; 22.5% respectively were positive using direct smear. Also our results correspond partially with outcomes of the findings obtained by [22] who mentioned that the rate of infection was 25%; 33%. Whereas these results were in contradicting with the findings obtained by with [19] in Eastern Turkey were 47.30%; 11.38 % were infected by *Babesia* sp., *Theileria* sp. respectively. The higher prevalence of *Theileria* might be related to the geographic distribution of the tick vectors or subclinical infections become chronic carriers of the piroplasm and hence, sources of infection for tick vectors [23].

Analysis of data revealed that, calves 46% were more infected by piroplasm as compared with adult animals 7.1%. Further analysis showed that the calves less than 2 years 33.3% were more prone to infection than their adult at aged 3-4 years 13.5% as in table 5. This in accordance with [24, 25] who reported highest prevalence in animals aged more than 3 years followed by the lowest prevalence in the less than one year age group. Also this result agreement with [26,27,28] who reported that the high prevalence of bovine theileriosis and babesiosis infection in small age groups (2 -12 months) and lower percentage of infection large age groups (more than 4 years). These results might be explained by the small age groups dyeing more susceptible to infection because does not keep immunity against the theileriosis and babesiosis infection while the old age groups due to the re-infection to one time or more developed a good immune defense that gives the animal resistance against infections [27,23].

In conclusions, the cattle in Al-najaf province are bearing the infection of piroplasmosis, also microscopic examination was not suitable in different cases, like detecting the carrier or chronic phases of piroplasmosis. However, it remains the most rapid confirmatory method for detecting this infection in acute phase of the disease. While PCR turned out to be a sensitive and accurate method for diagnosis in animals in the early phase of infection and mixed infections occur by DNA amplification. For recommended, PCR methods give us short-term outlook in observing of epidemiological condition calling for using microscopic examinations & serological methods. To illuminate recent epidemiological terrorization and to reduce the economic losses in cattle farming in Iraq, we expect as critical measures: extensive epidemiological survey making use of serology methods with molecular genetics methods, checking of distribution of tick vectors, availability of vaccination programs, and pathway of animal transports.

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