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Research article

Molecular detection of *Babesia bigemina* in ticks infesting water buffaloes (*Bubalus bubalis*) in Iraq

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Abstract

Babesiosis is consider as a hemoparasites disease transmitted through bites of infected ticks and is important tick-borne disease. A surveillance was carried out from June 2017 to February 2018 to identified ticks parasitize buffaloes. Hard ticks were 749 collected from different localities throughout AL-Qadisiyah, Babylon and Najaf Governorates for identification and classification. Stereomicroscope was used to identify the ticks based on their morphological features. DNA from 244 different individual tick species was extracted and PCR was performed for detection of Babesia bigemina in ticks parasitize buffaloes using specific primer targeting fragments of B. bigemina gene. 749 ticks of Ixodid species were collected from the buffaloes. Out of 320 studied buffaloes, 110 (34.38 %) were infested by 5 species of ticks. Hyalomma anatolicum was the most prevalent tick species (57.28%) followed by Hyalomma turanicum (17.36%), Hyalomma excavatum (10.95%), Hyalomma scupense(5.07%) and Rhipicephalus (Boophilus) annulatus (9.35%).An expected 1124 bp fragment of Babesia bigemina was generated in 23/70 (32.86%) of R.(Boophilus) annulatus. The results suggested that B. bigemina could be detected in the DNA extracted from R. (Boophilus) annulatus, confirming earlier reports as an important vector for B. bigemina. We conclude that B. bigemina is present in Ripicephalus (Boophilus) annulatus ticks in this area which detected by polymerase chain reaction (PCR). The present research work was the first attempt to determine the molecular prevalence of B. bigemina infection in ticks, in Iraq by using PCR.

Keywords: *Babesia bigemina*, Iraq, PCR ,Ticks**.**

Introduction

Buffalo (*Bubalus bubalis*) simply domesticated in Asia for meat and milk purpose. The phylogenetic archives defined that the buffalo initiated about (4000-5000 years) ago from China toward India (1). Parasitism is believed to be a main limitation in the growth of the livestock population including buffaloes. It causes health problems for domestic animals production (2). According to seasons, ticks are found actively through spring and summer months (3). There are different ticks, and tick-borne diseases transmitted by ticks to domestic animals (4, 5). Ticks are known to transmit the widest variety of pathogens such as viruses, bacteria, rickettsia, and protozoa, and producing diseases like fever, hemorrhagic babesiosis, anaplasmosis, theileriosis and ehrlichiosis, in animals (6). Tick and tick-borne diseases are worldwide problems for livestock health, and severity of these diseases depends on the host population, species of ticks, variation of progressive technologies for control procedures (5-7). Ticks cause either directly losses due to tick load, blood lossing, damage to skin and infection as a result of toxins, indirectly due to the weakness or mortality cause by tick-

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born diseases. Tick and Tick Born Diseases (TBDs) are caused an excessive economic importance in production of livestock (9).The annual global economic losses due to tick infestation have been expected US$14000-18000 million (8). Bovine babesiosis is mainly caused by *Babesia bovis* and Babesia *bigemina* in the tropic and sub tropic areas. Carrier animals play an important role in the transmission of Babesia species by ticks. These animals recover from infection when low number of erythrocytes remain infected with *Babesia* (10). Therefore, early and accurate diagnosis is essential to initiate proper treatment for the disease so application of molecular techniques for example PCR assays to study babesiosis epidemiology is very helpful. These examines have high sensitivity and specificity for the recognition of infection both in the ticks and vertebrate hosts (15, 16). However, the reports about the use of molecular methods for the finding of *B. bigemina* in tick are only few (17). Babesia is intraerythrocytic parasites cause disease of Babesiosis, which is one of the most common infections of free-living animals worldwide (13). All babesial parasites described to date are transmitted by ixodid ticks to their vertebrate hosts (11). The parasites replicate in the vertebrate hosts' red blood cells and are called piroplasms (12), Buffalo is one of the most important economic animals in Iraq, especially in the southern and middle region. Few studies about ectoparasites of buffalo in Iraq, (18) studied about the occurrence of hard ticks in some mammals. In other areas of Iraq, (19) recorded four species of Ixodids ticks in many areas in Wasit province in the of middle of Iraq. Buffaloes in Iraq has been affected by various factors as infestation by ticks that cause severe diminishing in production and /or population (14). Therefore, the purposes of this study were to distinguish the prevalence of infestation ticks on buffalo and to classify the ticks species collected and the present research was the first attempt to determine the molecular prevalence of *B. bigemina* infection in ticks, *R. (boophilus) annulatus* in Iraq by using PCR. The results of this study help understanding of disease epidemiology in the study districts, which will for provide planning of control strategies.

Material and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study.

Study area

Ticks collection was carried out from June 2017 to February 2018. The study was carried in AL-Qadisiyah, Babylon and Najaf governorates, Iraq. 320 buffaloes belong to 15 different herds spread over the study areas were randomly chosen.

Ticks collection

Specimens of Tick were collected from various sites on the buffaloes if present. They were placed separately in (1.5-ml) Eppendorf tubes containing (70% ethanol) to overcome contamination problem and transferred to the laboratory where they were examined in the Iraqi Natural History Research Center and Museum, University of Baghdad. Specimens of Tick were cleaned by washing double with distilled water then dried. They were identified under stereomicroscope to the species level according to (20, 21). The foremost identification features of the ticks are the shape of mouthparts, size, color, scutum, festoon, anal groove and legs. Identified sterilized ticks were stored individually, nymphs were stored in pools (2-4 specimens) in tubes contain 70% ethanol and stored at (-20ºC) prior to DNA extraction for detection of *B. bigemina* PCR.

Extraction of DNA

Extraction of DNA after the ticks were separated into pools. The collected tick

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specimens were homogenized in 200 µl of (Tissue lysis buffer) by using micro pestle. Then added 30µl of proteinase K and mixed by vortex and incubated at 60℃ for 1 hour. Genomic DNA was extracted from tick species according to company instructions by using gSYAN DNA mini extraction kit Insect sample protocol (Geneaid, USA), in elution steps of 100 µl for the best quantitative DNA result. The extracted tick genomic DNA was estimated by using NanoDrop spectrophotometer (THERMO, USA), which measured DNA concentration (ng/µL) and check purity of each DNA sample by analysis the absorbance at (260 /280 nm). After quantification, the DNA samples accordingly extracted were kept at -20°C till used for PCR analysis.

PCR amplification

For specific detection of PCR *B. bigemina* primer that used in this study were designed by (22) provided by (Bioneer company, Korea) shown in (Table 1) were used for ribotyping. PCR reaction was performed to obtain the 1124 bp amplified products over 35 cycles by 94ºC for 5 minutes , 94ºC for 30 seconds, 50ºC for 30 seconds, 72ºC for 45 seconds and completed with a final extension step of 7 minutes. at 72ºC. Finally the amplified DNA fragments were analyzed after electrophoresis on 1% agarose gel.

Statistical analysis

The data of the present study were carried out by Social Science Statistics and the Statistical Package. The valuation of relationship between the variables was studied by Chi-square test. Estimation was measured statistically significant if its (P value) was less than α level of significance of 0.05 (43).

Results

Ticks and ticks species

In the present study, results in (Table 2) and (Table 3) exposed that a total number of 749 ticks of Ixodid species were collected from 320 buffaloes (148 male and 172 female) from different localities in AL-Qadisiyah, Babylon and Najaf governorates. Tick infestation rate was (34.38 %) by five species of ticks, *Hyalomma anatolicum* was the most prevalent tick species (57.28%) followed by *Hyalomma turanicum (*17.36%), *Hyalomma excavatum (*10.95%), *Hyalomma scupense (*5.07%) and *Rhipicephalus (Boophilus*) *annulatus (*9.35%). No significance difference in rate of tick infestation between sex of host (P>0.05) and no significance difference (P>0.05) variation was detected between the locations of study (Table 3). Percentage of infestation with ticks according to months of study, significantly highest (p<0.05) infestation rate was observed in July (67.65 %), while tick infestation rate in December and January was (0 %) among the buffalo population (Table 3).

PCR detection of *Babesia bigemina* in ticks

PCR detection appear that only *Ripicephalus (Boophilus) annulatus* ticks, each females, males and nymphs, are infected by *Babesia bigemina* without significance differences (P>0.05) in infection rate by *Babesia bigemina* between males and females, furthermore no significance differences (P>0.05) in levels of infection between adult ticks and nymphs. Also the results show that *Hyalomma* spp. no infected by *Babesia bigemina (*Table 4).Set of oligonucleotide primers listed in (Table 1) were employed for detecting *B. bigemina* parasite in ticks. An expected 1124 bp fragment of *Babesia bigemina* was generated in 23/70 (32.86%) of *Rhipicephalus (Boophilus) annulatus* by PCR (Table 4). The results suggested that *B. bigemina* could be detected in the DNA extracted from *R. (Boophilus) annulatus,* (Figure 1) *confirming* earlier reports as an important vector for *B. bigemina*, while *Babesia bigemina* product was not detected in Hyalomma species in this area.

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Table (1): Primers used for amplify the DNA of *Babesia bigemina*

Amplicon	Nucleotide Sequence	Product length	Reference
18SrRNA gene	F TGGCGGCGTTTATTAGTTCG	1124bp	(22)
Babesia bigemina	R CCACGCTTGAAGCACAGGA		

Table (2): Prevalence of ticks species infested buffaloes

Tick species	No. of ticks	%
Hyalomma anatolicum	429	57.28%
Hyalomma turanicum	130	17.36%
Hyalomma excavatum	82	10.95%
Hyalomma scupense	38	5.07%
Rhipicephalus (Boophilus) annulatus	70	9.35%
Total no. of ticks	749	

***A Significant difference (P<0.05) was detected between prevalence of tick spp.**

Table 3prevalence of ticks infested buffalo according to location, sex and months

	Item	No.	Infested buffaloes	Frequency
Location	AL-Qadisiyah	115	42	36.52%
	Babylon	100	33	33%
	Najaf	105	35	33.33%
Total		320	110	34.38%
sex	Male	148	44	29.73%
	Female	172	66	38.37%
Total		320	110	34.38%
Months	June	45	25	55.55 %
	July	34	23	67.65 %
	August	39	22	56.41%
	September	35	20	57.14%
	October	28	11	39.28%
	November	38	5	13.16
	December	35	0	0
	January	32	0	0
	February	34	4	11.76
Total		320	110	34.38 %

***No significant difference (P>0.05) in prevalence between both sexes of buffaloes and studied areas. Significance difference (P<0.05) in prevalence among months of study.**

Table 4 Infection rate with *B. bigemina* in tick by PCR

Tick spp.	Total examined ticks no.	No. of examined males /no.+ve	No. of examined females /no.+ve	No. of examined nymph pools /no.+ve	Percentage of infection with *Babesia bigemina*
Ripicephalus(Boophilus) annulatus	70	36(9)	29(12)	5(2)	23/70 (32.86%)
Hyalomma anatolicum	75	41(0)	32(0)	2(0)	0/75(0%)
Hyalomma turanicum	41	20(0)	18(0)	3(0)	0/41(0%)
Hyalomma scupense	13	9(0)	4(0)	-	0/13(0%)
Hyalomma excavatum	25	11(0)	12(0)	2(0)	0/25(0%)
Total	224	117	95	13	23/224 (10.26%)

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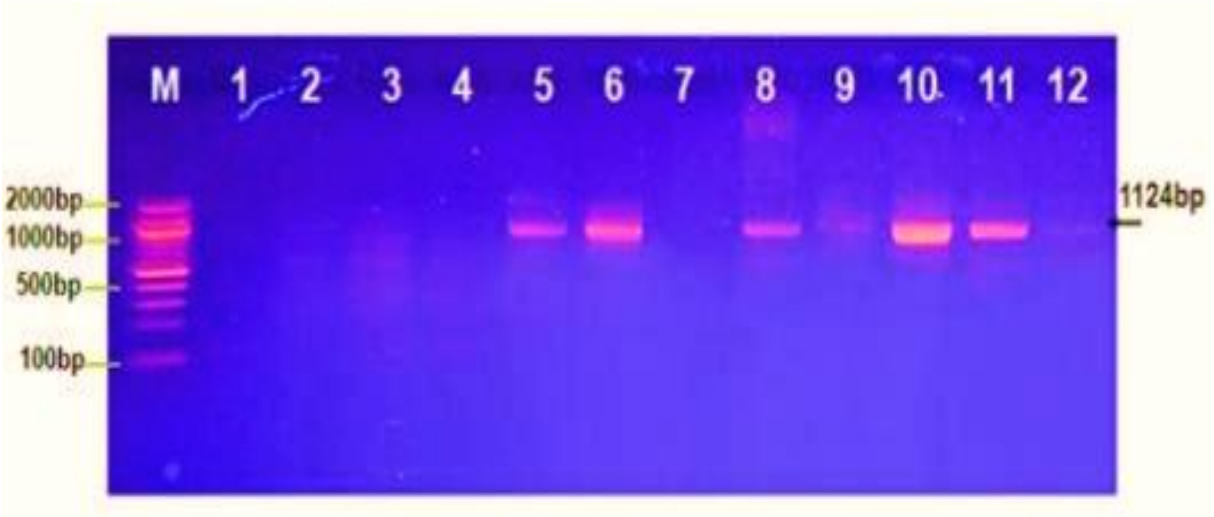

Figure(1): Agarose gel Electrophoresis image showing the PCR product analysis of gene of 18S ribosomal RNA in *B. bigemina* positive DNA of R. *(Boophilus) annulatus* ticks samples. Where M: Marker (2000-100bp) Lanes (5, 6, 8, 10 and 11) were positive samples at 1124bp product size.

Discussion

In the present study, the prevalence of Hyalomma species tick was highest followed by *R. (Boophilus) annulatus* (Table2). These result agreement with (23, 24). According to the current results, five species of Ixodid ticks were isolated, while (25) recorded three species only, *Hyalomma excavatum*, *H. turanicum* and *H. detritus* in Basrah governorate, in the south of Iraq. Ticks belong to genus Hyalomma are the dominant in the present study (26) referred to acclimation of Hyalomma sp. to the environment of the area and adaptation for hot or cold (27) observed that the buffaloes are usually infested with Hyalomma spp., while cattle are generally infested with Rhipicephalus (Boophilus) spp.. Buffaloes have access to mud for wallowing and have a not as much of thick hair coat, which might cause reducing of ticks and henceforth less infested with Boophilus species. (28) Reported Boophilus species distributed among domestic animals in Iraq, buffaloes, cows, sheep, goats and camels. (29) Showed that the differences in species may depend on the variety of weather climate. (30) Showed that ticks belong to genus Hyalomma are the dominant in the study, both in the number of species and individual ticks collected, followed by Rhipicephalus. The most prevalent species in the study was *H. anatolicum*. Tick infestation is a common problem of buffalo in Iraq. The present study conducted in three governorates in Iraq indicated that the percentage of buffalo tick infestation was 34.38% (Table-3), it was less than that of (24) who recorded the prevalence exceeded 50%. The severity and variation in prevalence of tick infestation in a specific geographical area perhaps depends on the diverse agro-climatic conditions, practices of animal husbandry, animals population, pasture management and host population (31).The association between host and vector ticks was depending on season for the growth of ticks. The variation between the results of the present study and earlier studies might be due to the differences in the geographical locations, climatic conditions, and methods of study, selection of sampling animal and breed of animal studied. Distribution of ticks on the hosts in the field influenced by various factors, such as the morphology of the host (length of the fur coat), the morphology of the tick (length of the hypostome) and immune responses of the host (39). In Rawalpindi and Islamabad area, (32) reported prevalence of Hyalomma species is

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26%, but Rhipicephalus species are not recorded. In all three governorates of the study, Hyalomma spp. were found to be the uppermost in prevalence compared with *R. (Boophilus) annulatus*. (33) Also showed that prevalence of Hyalomma spp. of ticks is higher than other species of the hard ticks in buffaloes. Therefore, information on the tick-borne pathogens prevalence in possible vector ticks is important for detection of tick-borne diseases. Rendering to sex of buffaloes in this study, the females had higher of rate of infestation than the males with no significant difference (P>0.05) in prevalence between both sexes of buffaloes, these result was in agreement with (34) who reported higher infestation rate of ticks in females than the males and they suggested that the hormonal stress carry more ticks. Amongst the buffaloes population examined a highest infestation rate significantly was detected in July while, without infestation was in December and January which is according to the previous reports(31, 35, 36) who found prevalence rate of ticks in June to August months. However, the highest prevalence of tick infestation in livestock was found in July, season and climate regulate tick population. The peak activity of *Rhipicephalus* species was mostly occurred during spring and summer. Also, adult ticks become active in the field with abundant livestock hosts (37). (29) Showed that the distribution of the causative protozoa is controlled by the geographical and seasonal distribution of the insect vectors that transmit them and the differences in species may depend on the variety of weather climate. Winter months recorded low rates of abundance, also similar clarifications have been reported by (38) who recorded the lowest prevalence of ticks abundance was in January. The results obtain by the present study shows that ticks have seasonal distribution. Based on PCR results, *B. bigemia* was detected in *R. (boophilus) annulatus*. This species is transmitted by nymphs and adults of R. Boophilus spp. ticks which considered as the usual vectors of babesiosis, these parasites pass through part of their life cycle in the invertebrate host. *R. Boophilus annulatus* is the major vectors of babesiosis (29). These results were consistent with the findings of other researchers (40, 41, 15). The results were different from those obtained by (42) who found that *Babesia* infection was detected in all of *Rhipicephalus* spp. except *R. annulatus*. It seems that low number of *R. annulatus* samples in Tavassoli survey may account for this difference. (42) Found that *Rhipicephalus* spp. as major vectors for bovine babesiosis in Iranian West and Iranian North-West. The observations from the present study may contribute to the increased understanding of epidemiology of ticks in Iraq. This may help in adopting tick control strategies. Therefore, further studies on tick prevalence are also suggested in other areas of Iraq to clear the knowledge of tick distribution in buffalo. The present research was the attempt to determine the prevalence of infection of *B. bigemina* in ticks, in Iraq, by using PCR.

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