Microscopic, serological and molecular detection of *Babesia bigemina* in buffaloes (*Bubalus bubalis*) in Wasit province- Iraq

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Abstract

The goal of present study was to detect an incidence of *Babesia bigemina* in apparently healthy buffaloes using of three different diagnostic assays. An overall of 179 buffaloes (*Bubalus bubalis*) in some rural regions in Wasit province /Iraq, were submitted for collection of blood samples during the period of December 2016 to February 2017. Initially, a thin blood smear slides were prepared and stained with Giemsa stain for microscopy, afterwards, the blood samples have been centrifuged for obtaining of serums and blood clots that examined by an indirect-ELISA and PCR, respectively. The overall results of testing all samples were revealed on 3 (1.68%), 46 (25.7%) and 21 (11.73%) positive animals by microscopy, indirect-ELISA and PCR, respectively. The significant differences ($P \leq 0.05$) were showed between the results of three diagnostic tests, and within the cross-classification values of microscopy to indirect-ELISA, microscopy to PCR, and indirect-ELISA to PCR.
Keywords: (*Babesia bigemina*, Buffalo, *Bubalus bubalis*, Serology, Molecular, Wasit)

Introduction:

Babesia bigemina is an intraerythrocytic protozoan that belonging to Babesiidae family of Apicomplexa phylum. This parasite is detected firstly by Vector Babes in 1888, and described as the more prevalent causative species for babesiosis disease in cattle and buffaloes (1, 2). It is accountable on great losses because of decreasing the animal production, using the approaches of controlling and treatment, with the influencing on trading international bovine animals (3).During acute phase, babesiosis is characterized by fever, anorexia, lethargy, haemoglobinuria diarrhea, as well as to emaciation, anemia and jaundice in more prolong severe cases (4). Afterword acute infections, the healthier animals frequently sustain subclinical infections that act as a source of babesiosis infection and latent vector for natural transmission (5). In general, babesiosis can be diagnosed in based upon the clinical signs of acute cases and can be demonstrated, microscopically, by staining of blood smears with Giemsa. Although, this method remains as a most suitable "*Gold Standard*" way for detection of acute infections, it's low in sensitivity and specificity in carrier animals and required for effective diagnostic tests to detect of *B. bigemina* (6). Several serological techniques are standarized for babesiosis detection and applied, extensively, in field studies (7). However, the cross-reactions and lacking of required specificity and sensitivity for diagnosis of carrier states (8). Recently, an indirect-ELISA has been developed in based on the ability of serum antibody to inhibit a monoclonal antibody directed against *B. bigemina* specific epitope. This assay is characterized by a high sensitivity, specificity and productivity (analysis of large number of animals in a shorter time) for determination of subclinical infections (9). On the other hand, PCR has proven to be high in its specificity and sensitivity more than other classical tests, with providing an active facilitating method in diagnosis of *B. bigemina* in carrier animals (10). Nonetheless and according to several studies, the combination of more than one technique in diagnosis of babesiosis can lead to a precise evaluation especially during the epidemiological investigations (8, 11). Hence, the goal of current study was to detect the prevalence of *B. bigemina* parasite in carrier buffaloes by using of three diagnostic

tools (blood smear's microscopy, serological indirect-ELISA and molecular PCR).

Material and Methods:

Sample of study

An overall 179 apparently healthy buffaloes, from both gender and more than 1 year age, were selected randomly from some rural areas at Wasit province during the period of December 2016 to February 2017.

From each animal, two samples of blood have been collected; the first from ear vein using of a heparinized capillary tube for preparation of blood smear slides, whereas, the second 6 ml blood sample were drained from jugular vein by a vacutainer EDTA tube (*AFMA, Jordan*) that transported to laboratory and directly centrifuged at 3000 rpm for 30 minutes.

The serum samples were kept in numbered 1 ml eppendorf microtubes, while the blood clot samples were kept in their tubes. Both sera and clots were saved at -20°C (8).

Study's Techniques

1. Microscopy: From each sample, two slides of blood smears (thin) were prepared, fixed by using of an absolute methanol alcohol (*Avantor, India*), Giemsa's stained (*SYRBIO, Syria*) and tested under 1000× of light microscopy (*Trinocular, MEIJI/Japan*) (12).

2. Serology: The serum samples were tested by an indirect-ELISA (*SVANOVA Biotech, Sweden*). All reagents and samples were equilibrated, diluted, incubated and rinsed according to manufacturer instruction. The results of controls and samples were measured at 405nm of optical density by using a microplate ELISA-Reader (*BioTek-USA*). Also, the calculation of mean OD values and interpretation of the results were explained in (Table 1):

Table (1): Calculation and interpretation of results

Calculation	$PP = \frac{\text{Mean OD value sample or Negative control}}{\text{Mean OD value Positive control}} \times 100$		
Interpretation	Control	OD Positive control	1-2.3
	Samples	PP Negative control	< 20
		PP Positive	≥ 40
		PP Negative	≤ 25

PP: Percent Positivity
OD: Optical Density

3. Molecular PCR: DNA was extracted from blood clots using of a commercial kit (*Bioneer, Korea*). GAU7 (F) 5'-GTTGGGTCTTTTCGCTGGC-3' and GAU8 (R) 5'-GCCAGCGAAA AGACCCAAC-3' primers were used for specific detection of *B. bigemina* (13). PCR reaction was performed to obtain the 685bp amplified product over 35 cycles, and processed in thermal cycler, (*MJ-BIO RAD/USA*), under the following conditions:

PCR Cycles	Temperature	Time
1 Cycle – Initial denaturation	94°C	2 Minutes
40 Cycles – Denaturation	94°C	30 Seconds
Annealing	55°C	30 Seconds
Extension	72°C	1 Minute
1 Cycles-Final extension	72°C	5 Minutes

Finally, the amplified DNA fragments were analysed after electrophoresis on 1% agarose gel (*Bioneer, Korea*). The amplified DNA products were stained with *Ethidium bromide* (0.4μg/ml) (*Bioneer, Korea*) and visualized under Ultra-violet (14).

Statistical data analysis

All obtained data concerning to the diagnostic assays were introduced, tabled and analysed using of two computerized Microsoft Office Excel (*2013*) and IBM/SPSS programs. The significant

differences were detected at a level of ($P \leq 0.05$), by application of descriptive statistics and Chi-square test (x^2) (15).

Results:

In this study, a totally of 179 healthy buffaloes were examined by light microscopy, indirect-ELISA, and PCR techniques which revealed on 3 (1.68%) positive blood smears, 46 (25.7%) seropositive and 21 (11.73%)positives, respectively, (Table 2). Cross-classification results of light microscopy to indirect-ELISA in a totally of 179 buffaloes, were showed that 3 (1.68%) of buffaloes were positives by both assays; and 43 (24.02%) were negatives microscopically and positives serologically, (Table 3). Cross-classification results of light microscopy to PCR in a totally 179 buffaloes, were showed that 3 (1.68%) of buffaloes were positives by both assays, and 18 (10.05%) were negatives microscopically and positives molecularly, (Table 4). Cross-classification results of indirect-ELISA to PCR technique in a totally 179 buffaloes, were showed that 20 (11.17%) of buffaloes were positives by both assays, 26 (14.53%) were positives serologically and negatives molecularly, and 1 (0.56%) was negative serologically and positive molecularly, (Table 5). Parasitological detection of *B. bigemina* within infected RBCs in blood smears slides stained with Giemsa was tested under 1000× oil immersion of objective lens light microscopy, (Figure 1). PCR product of *B. bigemina* positive isolates was showed by agarose-gel electrophoresis. While, Lane M represented the DNA marker (100-2000bp), Lanes (1-7) was referred to the positive samples at 681bp PCR product size at 1% agarose, 100 Volt and 80 Am for 1 hour, (Figure 2).

Table (2): Results of a totally 179 tested buffaloes by three diagnostic techniques

Diagnostic Techniques		Positive samples	Negative samples
1	Microscopy	3 (1.68%) ^{C}	176
2	Indirect-ELISA	46 (25.7%) ^{A}	133
3	PCR	21 (11.73%) ^{B}	158

Variation in vertical large letters referred to significant difference

Table (3) Cross-classification results of microscopy to indirect ELISA

	Indirect-ELISA Results		Total
Microscopy Results	Positive	Negative	
Positive	3 (1.68%) ^{Bb}	0 (0%) ^{Bb}	3
Negative	43 (24.02%) ^{Ba}	133 (74.3%) ^{Aa}	176
Total	46	133	179

Variations in large vertical and small horizontal letters referred to significant difference

Table (4): Cross-classification results of microscopy to PCR assay

	PCR Results		Total
Microscopy Results	Positive	Negative	
Positive	3 (1.68%) ^{Bb}	0 (0%) ^{Bb}	3
Negative	18 (10.05%) ^{Ba}	158 (88.27%) ^{Aa}	176
Total	21	158	179

Variations in large vertical and small horizontal letters referred to significant difference

Table (5): Cross-classification results of indirect ELISA to PCR assay

	PCR Results		Total
Indirect-ELISA Results	Positive	Negative	
Positive	20 (11.17%) ^{Ba}	26 (14.53%) ^{Bb}	46
Negative	1 (0.56%) ^{Bb}	132 (73.74%) ^{Aa}	133
Total	21	158	179

Variations in large vertical and small horizontal letters referred to significant difference

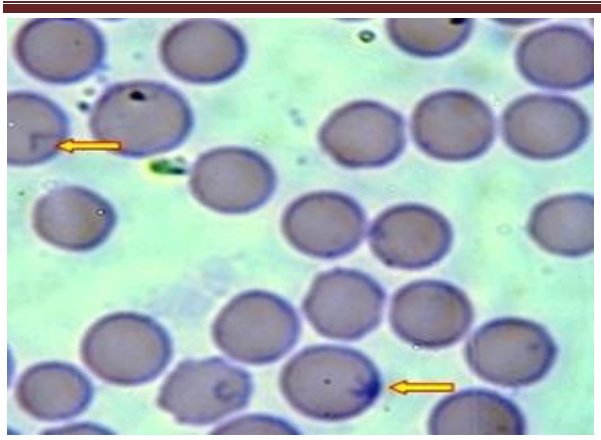

Fig. (1): *B. bigemina* within infected RBCs

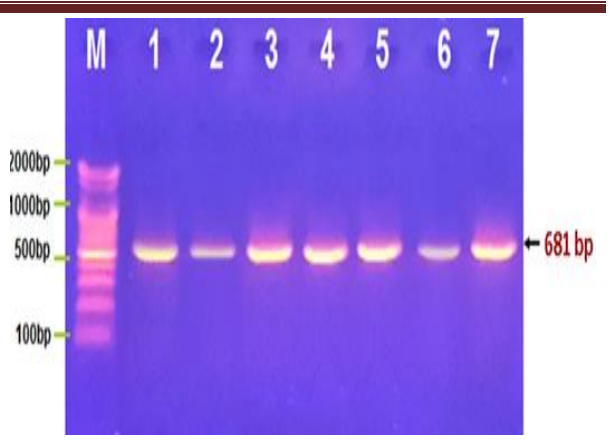

Fig.(2): Positive *B bigemina* samples by PCR

Discussion

Iraq, several studies have been directed toward diagnosis of *Babesia* sp. in cattle and buffaloes (16, 17, 18). In buffaloes (*Bubalus bubalis*), the present study was the first one that using three different diagnostic assays for evaluation of *B. bigemina* infections, involved the microscopy for stained blood smears, serology for serum samples by indirect-ELISA, and molecular detection of specific gene in whole blood samples using of PCR technique that revealed on 1.68%, 25.7% and 11.73% positive buffaloes, respectively. Worldwide, various methods were applied to detect the prevalence of bovine babesiosis in endemic areas, which reported variable results depending on the applied diagnostic test and stage of disease. In Iraq, though the livestock represented a great source for economy, the efforts still unmonitored and low benefits could be gained from these resources because of malnutrition, frequent infections, rottenly management systems, age old, lack of well-developed marketing fundamentals, inferior genetic makeup or unsuitable environments as bad farming and soil degradation. According to OIE, bovine babesiosis is classified under list of B because of their importance as a tick-borne infection that resulting in a significant morbidity and mortality (19). Carrier hosts infected with *Babesia* has been represented a challenge to be diagnosed because of a parasite might persistence in a few numbers in blood circulation (20). To overcome babesiosis economic losses, early, it's required a high sensitively techniques to proper diagnosis the carrier animals (21). Diagnostically, the microscopy is accounted as gold standard during detection the acute *B. bigemina* cases (22). Worldwide, the prevalence of bovine babesiosis in different countries by microscopic blood smears was varied from 0.6% to 32.2% (23), as reported in Turkey, 1.95% (23); in Pakistan, 9.9% (24); in Egypt, 10.76% (25); in Bangladesh, 14.1% (26); in Ethiopia, 16.9% (19); and in Brazil, 19-20.4% (27, 28). The method is simple to performance and inexpensive but required a high experience to detect of *Babesia* that appeared as a small pairs (2.5-3.5µm in diameter) of pear-like shaped merozoites insides the infected RBCs of animals, and differentiated it from other species (29). Also, the low sensitivity of test represented a great holdback that made the diagnosis more complication for detection of low parasitemia in a chronic stage of infection as well as in carrier animals and it dependable, only, where amounts of parasite in peripheral blood is highly enough to diagnose (25, 30). Hence, different serological methods were modified for detecting *Babesia* sp. among different phases of infection (7, 8).Recently, ELISA was used to be a confirmatory tool for results of microscopy and other serological tests because of its ability to detect of specific antibodies against *Babesia* sp. with high sensitivity, specificity, less subjectivity, and its capacity to be adapted to test large number of serum samples in short time. Moreover, the ability to couple the detection system to computerized automatic reader makes this assay a practical and in

(36, 37). Animals which not clinically ill may continue to infect the tick vector, so it can be used as a tool for epidemiological investigations. Moreover, an accurate early diagnosis of babesiosis in carriers is essential to overcome the economic losses (8, 21). With PCR, the prevalence of bovine *B. bigemina* was 1.34%, in Costa Rica (34); 7.1%, in Iran (38); 10.42%, in Egypt (25); and 16-34%, in Brazil, (27, 28). Several studies found out that the serological techniques could be detected a high numbers of diseased animals with babesiosis more than in molecular tests (11, 39, 40). These variations were in consistent with several previous studies. In these cases, the parasite might clear from peripheral blood, or the number of *Babesia* could be decreased for under-detectable level by PCR (41, 42). Advantages for integration of ELISA with PCR involved increasing of tests sensitivities during detection of hemoparasite, and the possibility of distinguishing of recent infections from established infection that the animal recovered from it (9, 42).Finally, this study were concluded a disability of blood smears microscopy in diagnosis of carrier buffaloes with *B. bigemina*, and the negative smears microscopy doesn't excluded a probability of infections. Also, study was established an ability of indirect-ELISA for providing an important data about an incidence of infections, whereas, PCR technique can be diagnosed the species of pathogen, accurately, without cross-reaction.

powerful tool for experimental and epidemiological studies (31). According to original data elicited from the serological studies that conducted in different regions of the world, the seroprevalence of bovine *B. bigemina* varies between 0.93-100% (23), such as in Egypt, 15.63% (25); in Turkey, 42.9% (23); in India, 56.11%-75.8% (32, 33); in Costa Rica, 59.1% (34); and in Brazil, 19% (35). However, several factors could be affecting on prevalence of bovine babesiosis such as ticks control, housing, feeding, farm size, stress, climate, age, and breed. Iraq is considered as one of the stable countries for babesiosis due to presence of pathogen throughout the year (16, 18).PCR has been proved to be sensitive in detecting of *Babesia* sp. in carrier animals, as well as in early acute phase of the infection, and hence, PCR is useful in evaluation the status of carriers that serve as disease's reservoirs for herds (36, 37). Animals which not clinically ill may continue to infect the tick vector, so it can be used as a tool for epidemiological investigations. Moreover, an accurate early diagnosis of babesiosis in carriers is essential to overcome the economic losses (8, 21). With PCR, the prevalence of bovine *B.*

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