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Microscopic-Serologic Survey of *Anaplasma marginale* Rickettsia in Buffaloes in Al-Qadisiyah and Babylon Governorates, Iraq

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Abstract

The aim of the present study was to detect the prevalence of *Anaplasma marginale* in buffaloes in two Iraqi governorates, Al-Qadisiyah and Babylon, by the microscopy as well as the competitive-ELISA that used firstly among the Iraqi buffaloes. A total of 184 buffaloes from both sexes of different age groups of animals were submitted for collection of blood samples to prepare the smears and sera. Overall results were revealed on 10.33% and 36.41% positive animals by microscopy and competitive-ELISA, respectively. In addition, positive rates by both tests were 7.61%; by microscopy only, 2.72%; and by competitive-ELISA only, 28.8%. In Al-Qadisiyah and Babylon governorates, respectively, 8.7% and 11.96% of microscopy samples, and 44.57% and 28.26% of competitive-ELISA were positives with significant differences (P>0.05). Regarding to age factor, the highest prevalence was detected by microscopy in young age group (1-3 years) was 14.15%, whereas by competitive-ELISA, it was 55% in adult buffaloes group (>3 years). Significant increases (P>0.05) in rates of infection were showed in females compared to males, respectively, by microscopy (11.18% and 4.35%) and competitive-ELISA (39.13% and 17.39%).

Keywords: *Anaplasma marginale*, Buffaloes, Microscopy, Competitive-ELISA, Iraq.

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INTRODUCTION

Anaplasma marginale is rickettsial intra-erythrocytic organism that causes bovine anaplasmosis in tropical, sub-tropical and temperate countries of the world, including Iraq; and being endemic in most animals of these regions[1,2]. *Anaplasma* that classified in *Alphaproteobacteria* class, *Rickettsiales* order of *Anaplasmataceae* family, is transmitted to cattle biologically by ticks and mechanically by flies and fomites[3,4]. Clinical disease is most notable in cattle, but other ruminants including water buffaloes can become persistently infected with *A. marginale*[5]. During acute anaplasmosis, *A. marginale* invades and multiplies within mature erythrocytes, leading to variable degrees of hemolytic anemia, fever, anorexia, weight loss, decreasing in milk production, reproductive problems and death in some cases[6, 7]. Recovering from acute phase results in persistent infected animal that serve as long-term reservoirs for transmission of infection within a herd[8]. The disease is a major constraint to bovine production because it affects dairy and beef domestic ruminants at any age resulting in high economic losses that estimated to be over 300 million dollars per year in United States [9, 10].

Microscopy is easy to perform, inexpensive, and considered as a "gold standard" test for confirming the acute phase of disease; however, its labor intensive and tedious for large numbers of samples, less sensitive, and impractical for routine testing of persistently infected ruminants as the bacterium is seldom detected in this phase[11, 12]. Hence, many serological techniques have been developed to detect specific IgM and IgG antibodies such as complement fixation test (CFT), card-agglutination (CAT), immunofluorescent antibody (IFAT) and enzyme-linked immunosorbent assay (ELISA)[13, 14]. Competitive-ELISA based on monoclonal antibody to recognize the major surface protein 5 (MSP5) of *A. marginale*, is used currently for diagnosis of bovine anaplasmosis[15]. It is highly accurate in diagnosis of acute and chronic infections with sensitivity and specificity that can reach to 95.6% and 98.6% of, respectively[16, 17].

The purpose of this study was to evaluate the prevalence *A. marginale* infections in buffaloes through microscopic diagnosis of intra-erythrocytic *A. marginale*-inclusion bodies in slides of blood smears, and serological detection of specific anti-A. marginale antibodies in sera, for first time in Iraq, by a competitive-ELISA. In addition, the association of positive samples obtained by both assays to some epidemiological factors (residence, age, sex) of study's buffaloes was evaluated.

MATERIAL AND METHOD

Study's samples

This study was performed in some rural regions related for two Iraqi governorates, Al-Qadisiyah and Babylon, during the period of March to August 2017. A total of 184 buffaloes from both sexes and different age groups were selected for the present study. From each animal, 10 ml of jugular venous blood was drawn and divided into two tubes (*AFMA, Jordan*); 3ml within an EDTA-anticoagulant tube to prepare of blood smear, and 7 ml within a free-anticoagulant tube that centrifuged at 3000 rpm for 15 minutes for sera. All sera were saved into numbered 1ml eppendorf tubes (*China*) and frozen at -20°C until be used[18].

Blood smears (Preparation and examination)

Acutely infected buffaloes with *A. marginale* were diagnosed by using a rapid staining of Diff-Quick set (Modified Giemsa). According to manufacturer instructions (*Vetlab Supplies, United Kingdom*), the slides of thin blood smears were prepared, fixed with fixative solution, stained with solution I then solution II, rinsed with distilled water and, finally, dried by air. By light microscope (*Trinoculr-MEIJI, Japan*), the stained slides were examined under oil immersion to detect the positive samples that having intra-erythrocytic corpuscles of *A. marginale* as small dark spots, of peripheral location, and ranging from 0.1-0.8 mm[19, 20].

Serological Survey

Competitive-ELISA was established for detection of specific IgG antibodies in persistently infected buffaloes with *A. marginale*. According to manufacturer instructions (*VMRD, USA*), the sera tested, and the results read using a microplate absorbance spectrophotometer reader (*BioTek*[λ]*, USA*) at an optical density (*OD*) of 650nm. The test validation has been made as the mean of negative control must have an *OD*>0.40 and ≤2.10, whereas, mean of positive control must have an inhibition of ≥30%. Regarding to interpretation of samples values, samples having ≥30% inhibition rate were considered positive.

Statistical analysis

All obtained data were tabled and classified using of Microsoft Office Excel program (*2013*), and analyzed by a computerized IBM/SPSS program ($V._{23}$) through application of descriptive statistics and Chi-square test (x^2). The significant differences between positive results of microscopic and serologic assays, and within residence, age, and sex factors of study's animals, were compared and detected at a level of $P \leq 0.05$[21].

RESULTS

Microscopic examination of blood smears obtained from 184 study's buffaloes revealed that 19 (10.33%) buffaloes were positives with specific intra-erythrocytic inclusion bodies of *A. marginale*, (Fig. 1). In addition, sera samples of 184 buffaloes were tested by a serologic competitive-ELISA that detected 67 (36.41%) seropositive buffaloes with anti-*A. marginale* IgG antibodies, (Table 1).

Table 1. Prevalence of *A. marginale* in an overall 184 buffaloes

Test	No.	Positives	Negatives
Light Microscope	184	19 (10.33%) ^{B}	165 (89.67%)
Competitive-ELISA		67 (36.41%) ^{A}	117 (63.59%)

Variation in large letters, vertically, refers to significant differences at level of $P \leq 0.05$

Table 2. Cross-classification results of microscopy and competitive-ELISA

Microscopy	Competitive-ELISA Positives	Competitive-ELISA Negatives	Total
Positives	14 (7.61%)^{Ba}	5 (2.72%)^{Bb}	19
Negatives	53 (28.8%)^{Ab}	112 (60.87%)^{Aa}	165
Total	67	117	184

Variation in large vertical and small horizontal letters refers to significant differences

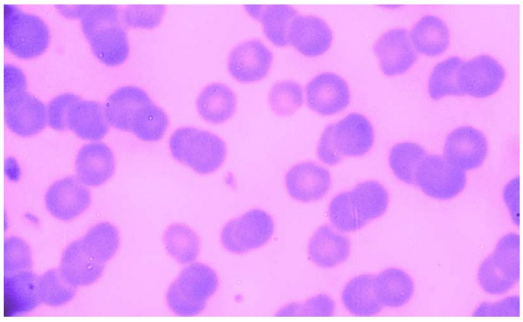

Fig. 1. Intra-erythrocytic *A. marginale* inclusion bodies

The results of (Table 2) showed that 14/184 (7.61%) of buffaloes were positives by both microscopy and competitive-ELISA, and 112/184 (60.87%) were negatives by both tests. On other hand, 5/184 (2.72%) of buffaloes were positives with microscopy, only; whereas, 53/184 (28.8%) were positives by competitive-ELISA, only.

Animals of this study were comprised 92 buffaloes from some areas of each governorate. Whereas, 11 (11.96%) and 41 (44.57%) of buffaloes were positives, respectively, by microscopy and competitive-ELISA in Al-Qadisiyah; 8 (8.7%) and 26 (28.26%) were positives by both tests, respectively, in Babylon (Table 3).

Among three age groups, positive buffaloes of microscopy and competitive-ELISA

Table 3. Association of positive *A. marginale* infections to residence factor

Residence	No.	Microscopy	Competitive-ELISA
Al-Qadisiyah	92	11 (11.96%) ^{Ab}	41 (44.57%) ^{Aa}
Babylon	92	8 (8.7%) ^{Ab}	26 (28.26%) ^{Ba}
Total	184	19	67

Variation in large vertical and small horizontal letters refers to significant differences

were distributed, respectively, as follow: in <1 year age group, 1/38 (2.63%) and 6/38 (15.79%); 1-3 years age group, 15/106 (14.15%) and 39/106 (36.79%); and in >3 years age group, 3/40 (7.5%) and 22/40 (55%), (Table 4).

Table 4. Association of positive *A. marginale* infections to age factor

Age	No.	Microscopy	Competitive-ELISA
<1year	38	1 (2.63%) ^{Cb}	6 (15.79%) ^{Ca}
1-3 years	106	15 (14.15%) ^{Ab}	39 (36.79%) ^{Ba}
>3 years	40	3 (7.5%) ^{Bb}	22 (55%) ^{Aa}
Total	184	19	67

Variation in large vertical and small horizontal letters refers to significant differences

Table 5. Association of positive *A. marginale* infections to sex factor

Sex	No.	Microscopy	Competitive-ELISA
Female	161	18 (11.18%) ^{Ab}	63 (39.13%) ^{Aa}
Male	23	1 (4.35%) ^{Bb}	4 (17.39%) ^{Ba}
Total	184	19	67

Variation in large vertical and small horizontal letters refers to significant differences

Among 161 female buffaloes, 18 (11.18%) and 63 (39.13%) were positives by microscopy and competitive-ELISA; while in 23 males, 1 (4.35%) and 4 (17.39%) were positives by both diagnostic methods, respectively, (Table 5).

DISCUSSION

According to FAO report in 1997, buffaloes are recognized as the "*Black gold of Asia*", however, few neglected studies have examined the occurrence of *A. marginale* among buffaloes if compared to other field animals[22]. In this study, the total rate of positive buffaloes with *A. marginale* was 10.33% by slides of blood smears microscopy and 36.41% by serological competitive-ELISA (Table1). In previous studies, the occurrence rate of *A. marginale* among buffaloes by blood smears microscopy was reported 5.71% in Iraq[23], 10.3% in Philippines (24), 4.29-22% in Pakistan[22, 25], 33.5% in South Africa[26], 59.3% in Egypt[27]; whereas, the seroprevalence of anti-*A. marginale* antibodies among buffaloes was 63% in Brazil[28], and 78.1% in Egypt[27]. Also, the study reported that 2.72% of buffaloes were positives, only, by light microscopy, which might be explained by the persistence of recent infection and IgG-antibodies were not developed, completely, to be detected by competitive-ELISA[29]; whereas, 7.61% of buffaloes were positives by both tests, which can be explained that these animals with acute infection and have high level of IgG-antibodies from previous exposure[30], at late stage of acute infection where the number of parasitemia decreased clearly and the immunity was increased, drastically[31], or presence of high immunity with severe infection[4]. Major surface protein (MSP5) is a highly conserved surface protein among different strains of *A. marginale*, which has been proven as effective diagnostic antigen and used in a competitive-ELISA[32]. MSP5-competitive-ELISA demonstrated a high sensitivity and specificity for determining the true-positive and true-negative animals (bovine, ovine, caprine, camelidae) in endemic areas[2, 33]. In addition, the test is excellent for detection of specific IgG antibodies in sera of naturally or experimentally infected hosts and in vaccinated animals, so that, it can be applied for eradication programs, regulation of interstate and international movement of reproductive field hosts[15,34]. Many studies reported that the test has an ability to detect of individually infected animals accurately. Hence, it can be utilized for epidemiological investigations where the infections expanding through the movement of infected animals into disease-free regions[35, 36].

Although, the worldwide seroprevalence of bovine anaplasmosis in buffaloes was reported to be less than that detected in cattle, the seropositive results of this study were higher than those reported previously in Iraqi cattle by[2, 37]. This could be attributed to that study's buffaloes were exposed for unsuitable environmental conditions such as stress factors and ticks[38]. Other reasons are the bad management systems that include problems in feeding, drinking, housing and disease control or medication, which leading to decrease or waning of immunity. In general, buffaloes can

play a role for harboring *A. marginale* and act as a potent carrier for other animals[39, 40].

In microscopy, although the positive prevalence of buffalo's *A. marginale* in Al-Qadisiyah (11.96%) was higher than reported in Babylon (8.7%) governorates; no significant differences (P£0.05) have been detected relatively between them, (Table 3). Whereas, the seroprevalence of infection by competitive-ELISA in Al-Qadisiyah (44.57%) much more than showed in Babylon (28.26%), and this could belong to variations in either owner's subculture, topography or to some risk factors such as stocking density, type of dipping, introduction of cattle to the farm, farm type, herd size, tick density, and dipping intervals[41, 42].

Positive results among different buffalo's age groups (Table 4) detected that the highest prevalence by microscopy was showed in young buffaloes (1-3 years age group), whereas by competitive-ELISA, it's seen in adults (>3 years age group). In young animals, these results might be explained by the age resistance and lack of maternal immunity gained by colostrums, which may last up 6 months to 1 year, hence more exposure for infections; whereas in adults, the seroprevalence of IgG-anti *A. marginale* antibodies was interpreted by the facts that the disease is of adults and the high titer levels of antibodies might be reflection for previous frequent multiple exposure to *Anaplasma* or recent infection [26, 27].

In relation to sex factor (Table 5), significant increases in A. marginale infections were detected in female buffaloes by both the microscopy and competitive-ELISA, which might belong to the low samples of study males in comparison to females, exposing of females to high stress conditions (gestation, parturition, milking), and/or that males received an attention more than females concerned to housing, feeding and medication[43, 44, 45].

CONCLUSION

The present study resumed that the prevalence of *A. marginale* in buffaloes have been increased, clearly, when compared to previous Iraqi studies; as well as, the seropositive results by competitive-ELISA were much more than reported by microscopy. In addition, differences in positivity among residence, age and sex factors could provide a benefit data for a futurism studies that recommended to be depended on competitive-ELISA or molecular techniques as a high sensitive and specific diagnostic methods.

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CONFLICTS OF INTERESTS

The author declare that there are no conflicts of interest.

AUTHORS' CONTRIBUTION

Author listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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