

Full Length Research Paper

Molecular and biochemical study of *Anaplasma marginale* in cattle in Wassit Province of Iraq

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This study was designed to investigate the prevalence of bovine anaplasmosis among cattle from various areas in Wassit governorate; the investigation was performed on 184 blood samples collected from suspected cattle suffering from fever (41°C), severe anemia, pale mucus membrane, progressive emaciation and drop in milk yield, including 85 male and 99 female cattle, aged from < 1 year to > 2 years, the samples were collected during the period of October 2012 - April 2013 from AL-Kut, AL-hayy, AL-Bashair, AL-Moufaqia and AL-Noamania areas to investigate antibodies against *Anaplasma* parasite by using indirect enzyme-linked immunosorbent assay (ELISA) test and to determine the species of genus *Anaplasma* by using RFLP-PCR technique and by also measuring some biochemical parameters to indicate the effect of the disease on liver function. The results of ELISA test showed that the rate of infection was 13.04%, the rate of infection was different between age groups and were 8, 11.25 and 16.45% in ages < 1, 1-2 and 2 - 3 years, respectively. The study revealed that females were given higher percent of infection 14.14% than males 11.7%, there is no significant differences under $p > 0.05$ according to age groups and sex. The highest rate of infection was recorded in AL-Kut, 17.14% followed by AL-hayy, 14% and AL-Bashair, 10% and the lowest rate was recorded in AL-Noamania, 8.33% and AL-Moufaqia, 5%; the study showed significant differences in incidence of disease between study districts and area in Wassit governorate at $p > 0.05$. The most sensitive method for the diagnosis of anaplasmosis is the method of polymerase chain reaction, DNA extraction was performed only on 24 blood samples which were positive for *Anaplasma* spp. by ELISA test, the extracted DNA from blood cells were analyzed by PCR and PCR-RFLP technique using primers derived from 16S rRNA gene and restriction endonuclease *Bst*1107I enzyme which can recognize the sequence (GTATAC) in corresponding PCR product of *Anaplasma marginale* and cut it in the position 68 and 509, whereas the used restriction enzyme cannot cut the corresponding PCR product of other *Anaplasma* spp. and the result was 20 from 24 which were positive for *Anaplasma* spp. by PCR and 18 from 20 was positive for *A. marginale*. The results of liver enzymes activity showed significant increase in serum AST, ALT, CK and TBIL level in infected cattle (96.8±0.97, 42±0.52, 406±2.06 and 0.95±0.24, respectively) as compared to the control (65.5±1.26, 21.4±0.45, 142±14.17 and 0.27±0.05, respectively).

Key words: *Anaplasma marginale*, indirect enzyme-linked immunosorbent assay (ELISA), RFLP-PCR, biochemical parameters.

INTRODUCTION

Bovine anaplasmosis is one of the important hemoparasitic tick-borne disease of cattle and other ruminants with tropical and sub-tropical regions of the world, caused by intraerythrocytic parasite of the genus

Anaplasma (order: Rickettsiales; family: Anaplasmataceae) (Dumler et al., 2001). Based on location within the infected erythrocyte, two species of *Anaplasma* that infect cattle and cause bovine

Table 1. Oligonucleotide sequences that were used for PFLP-PCR.

Primer	sequence	PCR product size
16S rRNA	F AGAGTTTGATCCTGGCTCAG	577 bp
	R GTTAAGCCCTGGTATTTAC	

anaplasmosis have been described, *Anaplasma marginale* (Theiler, 1910) and *Anaplasma centrale* (Theiler, 1911). The most common etiological agent is *A. marginale* which cause acute anaplasmosis and responsible for severe morbidity and mortality in temperate tropical and subtropical regions worldwide (Palmer et al., 2000). According to Theiler (1911), *A. centrale* is less pathogenic to cattle than *A. marginale* but, most importantly, gives resistance against the latter; hence it is used for the preparation of live vaccine strains, assuring immunological protection against bovine anaplasmosis, such vaccines are produced in Africa, Australia, Latin America (Kocan et al., 2003). Bovine anaplasmosis occurs in tropical and sub-tropical regions of the world including 40 states of the USA, south and Central America, Asia, Africa, southern Europe, Middle East and Australia (Cynthia and Susan, 2008). The infectious agent is transmitted either biologically by ticks or mechanically via contaminated mouth part of biting insects or by contaminated fomites such as needles, castrating knives, ear taggers and other surgical instruments (Ewing et al., 1997).

Bovine anaplasmosis can be seen at any age group in cattle, however, the severity of disease and death rate increase with the advance in age, with clinical anaplasmosis being more commonly in cattle older than 1 year of age (Kocan et al., 2003). Acute anaplasmosis is characterized by a progressive hemolytic anemia associated with fever, weight loss, abortion, decreased milk production and in some cases death of infected cattle (Kocan et al., 2000). Animals that recover from acute anaplasmosis become persistently infected with *A. marginale* and serve as reservoir and source of infection within the herd (Aubry and Geale, 2011). The diagnosis of bovine anaplasmosis is performed routinely by Giemsa stained blood smears which can indeed be used as a suitable method to detect *Anaplasma* in the animals clinically suspected for acute diseases, but it is not applicable to the determination of pre-symptomatic and carrier animals (Carelli et al., 2007). Therefore, several serological tests have been used to measure *Anaplasma*-specific antibodies, including the complement fixation test, card agglutination test, indirect fluorescent antibody test and enzyme-linked immunosorbent assay (ELISA) (Goff et al., 1990). Unfortunately, because of antigen

cross reactivity, these tests do not discriminate between different *Anaplasma* species (Dreher et al., 2005). PCR is an alternative diagnostic technique, which offers a high degree of sensitivity and specificity, and has been developed to identify *A. marginale* DNA in the blood of infected animal even if present in very low numbers (Carelli et al., 2007).

MATERIALS AND METHODS

Blood sample collection

From October 2012-April 2013, 184 venous blood samples were collected from cattle suspected to have anaplasmosis aged from less than 1 year to 2 - 3 years and based on the clinical manifestation of severe anemia and jaundice from districts and the areas of Wasiit governorate (AL-kut, AL-hayy, AL-Bashair, Al-Moufaqia and Al-Noamania). 5 ml of blood samples were collected from the jugular vein into 2 vacutainer tubes, one with anticoagulant for PCR, and the other without for the ELISA.

Serum preparation

The serum was separated by centrifugation at 3000 rounds per minute for 10 min, then aspirated carefully by pipette into dry, sterile and labeled test tubes, which were stored frozen then transported under cold conditions to the laboratory of Al-Karama hospital, where indirect ELISA test was conducted for detection of *A. marginale* antibodies using an *A. marginale*-Ab ELISA Kit (Svanova Biotech AB, Sweden).

Primers

The 16S rRNA primers used in REFLP-PCR for genotyping of *Anaplasma* spp. were designed by Noaman et al. (2009) and provided by Bioneer company, Korea and are listed in Table 1.

Serological analysis

A. marginale-Ab ELISA Kit (Svanova Biotech AB, Sweden), was used to detect specific antibodies against *A. marginale* in bovine serum samples. This technique was done according to the manual.

Molecular analysis

PCR followed by RFLP was performed for detection and genotyping of *Anaplasma* spp. in blood samples that were give positive results

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Table 2. Seropositivity rate of bovine anaplasmosis based on ELISA.

No. of examined cattle	No. of ELISA positive cases	Infection rate (%)
184	24	13.04

Table 3. Infection rates according to age based on ELISA.

Age group	No. of animals examined	No. of ELISA positive cases	Infection rate (%)
Less than 1 year	25	2	8a
1-2 years	80	9	11.25a
Higher than 2 years	79	13	16.45a
Total	184	24	13.04

Similar letters refers to the non-significant differences between ages ($p < 0.05$).

Table 4. Infection rates according to sex of animals based on ELISA.

Cattle	No. of animals examined	No. of ELISA positive cases	Infection rate (%)
male	85	10	11.76a
Female	99	14	14.14a
Total	184	24	13.04

The similar letters refers to the non-significant differences between sexes ($p < 0.05$).

Table 5. Infection rates according to location based on ELISA.

Area	No. of animals examined	No. of ELISA positive cases	Infection rate (%)
Al-Kut	70	12	17.14a
Al-hayy	50	7	14a
Al-Bashair	20	2	10ab
Al-Noomania	24	2	8.33ab
Al-Moufaqia	20	1	5b
Total	184	24	13.04

Similar letters refers to the non-significant differences while different letters refers to the statistically significant differences among areas ($p < 0.05$).

in ELISA. This technique was based on the digestion of 16S rRNA gene by restriction endonuclease (*Bsf1107I*) and was carried out according to methods previously described by Noaman et al. (2009).

Genomic 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 the company's instructions.

Biochemical tests

AST, ALT, CK and TBIL were measured using biochemical test kits and according to the manufacturer's instructions.

Statistical analysis

Chi-square (χ^2) and t-test were used to detect statistically significant differences between age groups, sex and study area of data prevalence of disease and the effect of other factors. The

differences were considered statistically significant at $P > 0.05$ (Al-Rawi, 2000).

RESULTS

Prevalence of bovine anaplasmosis in cattle using ELISA test

Out of 184 serum samples collected from suspected cattle examined by ELISA, there were 24 (13.04%) positive cases (Table 2). The study showed that higher infection rate was recorded in animals aged 2-3 years, 16.45% (13/79), while the lowest infection rate was in animals less than 1 year of age, 8% (2/25) (Table 3). Also, the study revealed that females were given higher percent of infection, 14.14% (14/99) than males, 11.7% (10/85) (Table 4). Higher percent of infection was record in AL-Kut, 17.14% (12/70), while the lower percent was record in Al-Moufaqia, 5% (1/20) (Table 5).

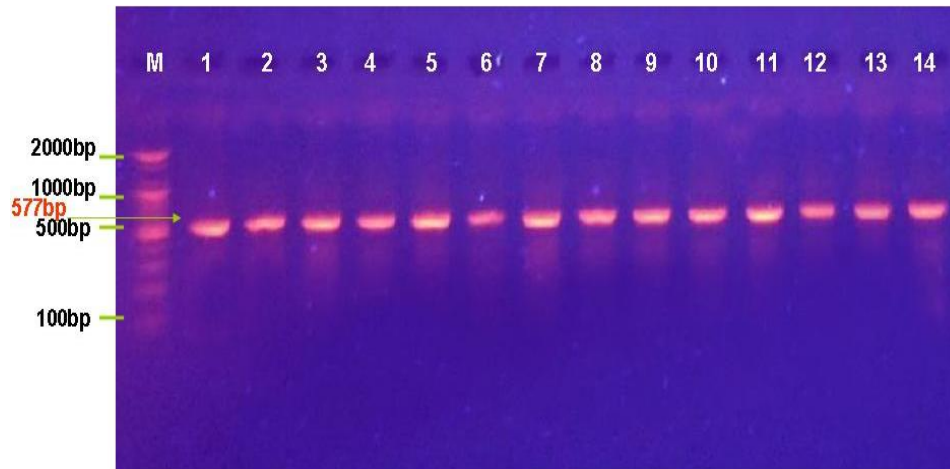


Figure 1. Agarose gel electrophoresis image that shows the PCR product of 16S the rRNA gene of *Anaplasma* spp. isolated from blood samples. Where M: marker 100 bp, lane (1-14) positive blood samples at expected size of 577 bp PCR product.

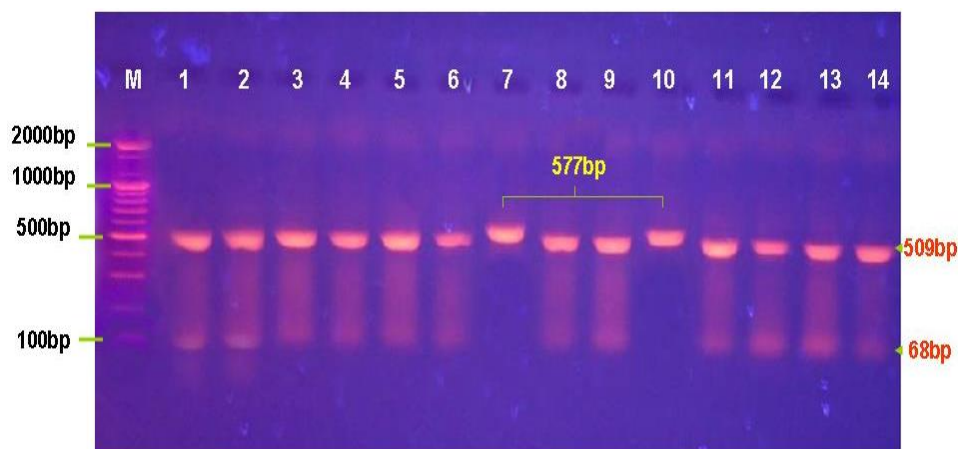


Figure 2. Agarose gel electrophoresis following restriction digestion of the 577 bp amplicon of the 16S rRNA gene of *Anaplasma* spp. using the restriction endonuclease (*Bst*1107I). Where M: marker 100 bp, (lanes 1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13 and 14) positive *Anaplasma marginale* that gave cut at 509 and 68 bp, while lanes 7 and 10 was negative for RFLP PCR that was still kept at 577 bp PCR product.

Bovine anaplasmosis according to RFLP-PCR technique

This technique was used to determine *Anaplasma* species present in the study area. PCR of the DNA isolated from blood samples showed that 20 out of the 24 blood samples were *Anaplasma* spp. positive and resulted in an expected product of 577 bp (Figure 1).

The restriction endonuclease *Bst*1107I when added to *A. marginale* 16S PCR products resulted in DNA fragments with the expected sizes of 509 and 68 bp. Thus, 18 out of 20 blood samples were confirmed to have *A. marginale* (Figure 2).

DISCUSSION

Prevalence of bovine anaplasmosis according to the ELISA test

In the current study, the prevalence of antibodies against *A. marginale* in cattle from Wassit province was investigated using an ELISA. The results demonstrated that these ruminants are exposed to this hemoparasite in the region studied and this result is agreement with worldwide distribution of anaplasmosis in cattle (Kocan et al., 2010). The results of this study revealed that the rate of infection in cattle was 13.04% and was comparative

with other studies done in Iraq, this result was higher than the result recorded by Ameen et al. (2012) who showed that 9.09% of cattle in Erbil were infected with anaplasmosis but lower than the result (30.4%) which was reported by Al-Mossawy (2012) in Al-Diwanyia governorate, this difference in results is due to the differences in weather conditions and geographic distribution of tick vectors between Erbil and AL-Diwanyia with Wasit governorates. Specifically, the weather is cooler in the north which results in low distribution of tick vectors available to transmit the disease, while AL-Diwanyia is a big agricultural area with concentrated animal husbandry especially cows. The warm climate in this area allows for the presence of the tick vectors in huge number, thus a higher prevalence of infection. Regarding age wise prevalence, the results of this study revealed that the highest rate of infection was in cattle of over 2 years old (16.45%), while the lowest rate was in calves less than 1 year (8%). However, the differences between groups were not statically significant. This result agrees with many similar studies (Al-Mossawy, 2012; Chowdhury et al., 2006; Al-Khaledi, 2008) that reported that the highest prevalence of anaplasmosis is in cattle of 2 years of age and over. Calves from immune dams receive temporary protection from the colostrum which prevents anaplasmosis, this protection lasts about 3 months and in most cases, is followed by age resistance which lasts until the animals are approximately 9-12 months of age. The age resistance in calves gradually wanes after 1 year of age and these animals become increasingly susceptible to the disease (Kocan et al., 2000; Tassi et al., 2002).

In relation to the sex wise prevalence, the results of the study indicated that females are more susceptible than male to anaplasmosis infection with the rate 14.14 and 11.7%, respectively but the statistical analysis show no significant differences. The higher prevalence of anaplasmosis in female animals may be due to the fact that contaminated needles are commonly used for injecting drugs for milk let down (Kocan et al., 2010).

The immunosuppression in advanced pregnancy and or lactation in high producing animals are the possible reasons for the higher prevalence of *A. marginale* in female cattle. Also, differences in management practices in which male receive high quality feed, treatment with ectoparasitic drugs as well as housing few cohorts in relatively clean conditions yard in order to reach high body weight for sale, all can lead to decrease exposure of males to *A. marginale* (Al-Mossawy, 2012).

The present study showed significant differences in incidence of disease between study districts and area in Wasit governorate, this difference may be attributed to the differences in climatic conditions and intensity of ticks' infestation in the areas, the highest rate of infection was record in AL-Kut, 17.14% followed by AL-hayy, 14% and AL-Bashair, 10%. The areas with high percentage of infection are agricultural areas where available animal

husbandry and a wet and warm climate are conducive for tick transmission, which are the key factors in *A. marginale* transmission. These areas are situated near Iran in which many casualties among the herds of cows was recorded and from where there is possible entry of infected animals into the country through trade or animal movement. The lowest prevalence was 8.33 and 5% in Noamania and Mouafaqia, respectively. This may be attributed to the desert climate of these two regions in which animals and tick vectors are fewer.

Prevalence of bovine anaplasmosis according to RFLP-PCR method

In the present study, DNA was extracted from 24 blood samples which were positive for *Anaplasma* by ELISA. The extracted DNA from blood cells was analyzed by PCR and RFLP using primers derived from 16S rRNA gene and restriction endonuclease Bst1107I. The restriction endonuclease Bst1107I only recognizes the sequence (GTATAC), which is specific for *A. marginale* (Noaman and Shayan, 2010). Based on the PCR, 20 of 24 samples were positive and this suggested that the rest 4 negative sample was collected from cattle in the late stage of infection when all parasite's DNA was faded. Analysis of all 20 *Anaplasma* positive PCR products with the restriction endonuclease Bst1107I showed that 18 PCR products could be cut in two expected DNA fragments with 509 and 68 bp in length, respectively, and this confirm diagnosis that cattle in this study were infected with *A. marginale*. The failure of restriction endonuclease cutting in the remaining two samples suggests these animals may have been infected with *A. centrale* or *A. bovis*. This simple PCR method based on 16S rRNA gene flowed by RFLP-PCR is able to rapidly discriminate between *A. marginale* and another species within the genus *Anaplasma* that infect cattle. The results of this study are in agreement with the results reported by Noaman and Shayan (2010) in Iran, which revealed the possibility of developing a new PCR-RFLP method based on 16S rRNA gene able to differentiate between *A. marginale*, *A. centrale* and *A. bovis* that infect cattle.

Biochemical parameters

Concerning the effect of *A. marginale* infection on activity of liver enzymes, the obtained results of the study revealed significant increase in serum AST and ALT in infected cattle when compared with healthy cattle. These results were in agreement with other studies reported by Allen et al. (1981) and (Turgut 2000). Allen et al. (1981) stated that the increase in enzyme activity may be attributed to severe anemia that leads to hypoxic and liver damage. Also, massive hemolysis may occur which in conjunction with hypoxia may lead to hepatic cell degeneration and glomerular dysfunction leading to increase in AST and ALT enzymes. In the present study,

Table 6. Levels of biochemical parameters in infected and control.

Parameter	Control (n = 10)	Infected cows (n = 10)
AST	65.5±1.26a	96.8±0.97b
ALT	21.4±0.45a	42±0.52b
CK	142±14.17a	406±2.06b
TBIL	0.27±0.05a	0.95±0.24b

Values were shown as Mean ± SE. The different letters denote the statistically significant differences at $p > 0.05$.

the results revealed an increase in creatine kinase (CK) and total serum bilirubin concentration in infected cattle as compared to healthy cattle. The result agrees with Coskun et al. (2012) which explain that this increase may have been caused by muscular trauma as a result of recumbency due to anaplasmosis (Sandhu et al., 1998) and Hofmann-Lehmann et al. (2004) mentioned that anaemia results from extra-vascular phagocytosis and hemolysis of parasitized erythrocytes by the reticulo-endothelial system may cause increase in serum bilirubin concentration and may be recognized by yellow discoloration of the plasma. Furthermore, Manna (1990), Omer et al. (2003) and Khan et al. (2011) reported that hyperbilirubinemia in anaplasmosis is due to excessive destruction of RBCs and hepatic dysfunction (Table 6).

Conflict of interests

The authors did not declare any conflict of interest.

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