

Some molecular and sero – prevalence study of *Anaplasma marginale* in cattle in Wassit province

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Abstract

The 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 including (85) males and (99) females, aged from < 1year to > 2 years. Samples were collected during the period from October 2012 to April 2013, from AL-Kut, AL-hayy, AL-Bashair, AL-Moufaqia and AL-Noamania areas for investigations about the presence of antibodies against *Anaplasma* parasite by using indirect ELISA test and to determinate the species of genus *Anaplasma* by using RFLP-PCR technique. The suspected cattle suffering from fever (41°C), severe anemia, pale mucus membrane, progressive emaciation and drop in milk yield. The results of ELISA test showed that the rate of infection was 13.04 %; the rate of infection was different between age groups and was 8 %, 11.25 % and 16.45 % in ages < 1year, 1-2 year and 2 - 3 years respectively. The study revealed that females were given higher percent of infection 14.14 % than males 11.7 %, and there were 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 the studied districts and areas in Wassit governorate under $p < 0.05$. The most sensitive method for the diagnosis of Anaplasmosis was the method of polymerase chain reaction. The DNA extraction was performed only on 24 blood samples which 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 recognizes the sequence (GTATAC) in corresponding PCR product of *A. 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 out of 24 was positive for *Anaplasma spp.* by PCR and 18 out of 20 was positive for *A. marginale*.

Key words: ELISA test, PCR, prevalence, *Anaplasma marginale*, cattle.

دراسة مصلية و جزيئية عن مرض الانابلازموسس في الابقار في محافظة واسط

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الخلاصة

صممت الدراسة للبحث في انتشار مرض الانابلازموسس بين قطعان الابقار في مناطق مختلفة من محافظة واسط وقد اجري البحث على 184 عينة دم جمعت من ابقار مشكوك في اصابتها وشملت (85) ذكر و (99) انثى تتراوح اعمارها بين اقل من سنة الى اكثر من سنتين ، جمعت العينات خلال الفترة من تشرين الثاني 2012 الى نيسان 2013 من مناطق الكوت ، الحي ، البشائر، الموقبية و النعمانية للبحث عن الجسام المضادة الخاصة لطفيلي الانابلازما بواسطة طريقة الاليزا الغير مباشر ولتحديد النوع الخاص بجنس الانابلازما بواسطة تقنية تفاعل سلسلة البلمرة (PCR) وتفاعل سلسلة البلمرة نوع تقييد طول الجزء متعدد الأشكال (RFLP-PCR). الابقار المشكوك بها تعاني من الحمى (41م°) ، فقر دم شديد ، شحوب الاغشية المخاطية ، هزال شديد وانخفاض في انتاج الحليب. أظهرت نتائج فحص الاليزا ان نسبة الاصابة (13.04%) ، وكانت نسبة الاصابة مختلفة بين مجاميع الاعمار فكانت بنسبة (8%) ، (11.25%) و(16.45%) بعمر

أقل من سنة ، 1 - 2 سنة ومن 2 - 3 سنة على التوالي. أظهرت الدراسة نسبة الإصابة في الإناث (14.14 %) أعلى منها في الذكور (11.7 %) ، إلا أنه لا يوجد فرق معنوي تحت مستوى احتمال 5%. أعلى نسبة إصابة سجلت في الكروت 17.14% تتبعها الحي 14% و البشائر 10% أما أقل نسبة إصابة سجلت في النعمانية 8.33% و الموقية 5% مع فرق معنوي تحت مستوى احتمال 5%. بينت النتائج ان الطريقة الأكثر حساسية في تشخيص الانابلازموسس هي تفاعل سلسلة البلمرة (PCR) حيث تم استخلاص الحمض النووي DNA من 24 عينة فقط والتي كانت موجبة لفحص الاليزا وقد تم تحليل الحمض النووي المستخرج بواسطة تقنية PCR و (RFLP_PCR) باستخدام بادئات مستمدة من 16S rRNA و انزيم القطع endonuclease Bst1107I الذي يميز تتابع القواعد النتروجينية (GTATAC) لنواتج PCR الخاصة *A. marginale* ويقوم بقطعها بالموقع 68 و 509 في حين ان الانزيم لا يقطع نواتج PCR الخاصة بالأنواع الاخرى لطيفلي الانابلازما وكانت النتيجة 20 من 24 عينة موجبة لل *Anaplasma spp.* و 18 من 20 موجبة للنوع *A. marginale*.

الكلمات المفتاحية: اختبار الاليزا ، اختبار تفاعل سلسلة البلمرة ، الانابلازموسس ، الإبقار

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 genus *Anaplasma* (order: Rickettsiales, family: Anaplasmataceae) (1). Based upon location within the infected erythrocyte, two species of *Anaplasma* that infect cattle and cause bovine Anaplasmosis have been described, *A. marginale* and *A. centrale* (2, 3). 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 (4). According to Theiler (3) *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, and Latin America (5). Bovine anaplasmosis occurs in tropical and sub-tropical regions of the world including 40 states of USA, south and central America, Asia, Africa, southern Europe, Middle East, and Australia (6). The infectious agent 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 (7). 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, clinical anaplasmosis is more commonly in cattle older than 1 year of age (5). Acute

anaplasmosis characterized by a progressive hemolytic anemia associated with fever, weight loss, abortion, decreased milk production, and in some cases death of infected cattle (8). Recovered animals from acute anaplasmosis become persistently infected with *A. marginale* and serve as reservoir and source of infection within a herd (9). Diagnosis of bovine anaplasmosis performed routinely by Giemsa stained blood smears which can be indeed used as a suitable method to detect *Anaplasma* in the animals clinically suspected for acute diseases, but it is not applicable for the determination of pre-symptomatic and carrier animals (10). Therefore, several serological tests have been used to measure *Anaplasma*-specific antibodies, including the complement fixation test, card agglutination test, indirect fluorescent antibody test, enzyme-linked immunosorbent assay (ELISA) (11). Unfortunately, because of antigen cross reactivity, these tests do not discriminate between different *Anaplasma* species (12). Molecular methods include PCR technique, with a high degree of sensitivity and specificity, have been developed to identify *A. marginale* DNA in the blood of infected animal even in very low numbers (10). For that the study aimed to 1- Investigate about Anaplasmosis in cattle by Enzyme-Linked ImmunoSorbent Assay (ELISA) technique and determine the effect of age and sex of animals and study area on prevalence rate of infection. 2-To determinate the species of *Anaplasma marginale* or *A. centrale*, by use of PCR-RFLP method.

Materials and methods

Blood Sample collection:

From October 2012 to April 2013, a 184 venous blood samples were collected from suspected cattle aged from less than 1 year to 2-3 years and based on the clinical manifestations of severe anemia and jaundice from districts and the areas Wasiit governorate (AL-kut, AL-hayy, AL-Bashair, Al-Moufaqia, and Al-Noamania). Blood samples were collected from jugular vein into 2 vacutainer tubes each contain 5ml of blood, one with anticoagulant for PCR test, labeled and stored in freezing condition inside the refrigerator, and the other without anticoagulant, and preparation of serum was done immediately for ELISA test.

Serum preparation:

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

Primers:

The 16S rRNA primers which used in REFLP-PCR for genotyping of *Anaplasma* spp. were designed by (13) and provided

from Bioneer company, Korea as following (Table 1).

Table (1): Oligonucleotide sequences that were used for PFLP-PCR

Primer		Sequence	PCR product size
16S rRNA	F	AGAGTTTGATCCTGGCT CAG	577bp
	R	GTAAAGCCCTGGTATTT CAC	

Serological analysis:

A. marginale-Ab ELISA Kit (Svanova Biotech AB, Sweden), was used to detect specific antibodies against *Anaplasma marginale* parasite in bovine serum samples. This technique was done according to method described by company instructions.

Molecular analysis:

RFLP PCR Technique was performed for detection and genotyping of *Anaplasma* spp. in blood samples that collected from suspected cases of cattle, this technique was based on the digestion of 16S rRNA gene by restriction endonuclease (*Bst*1107I) and the method was carried out according to method described by (13).

Statistical analysis:

Chi-square (X^2) and t-test were used for detect statistical difference of data prevalence of disease and the effect of other factors. The differences were considered statistically significant at $P < 0.05$ (14).

Results

Prevalence of Bovine Anaplasmosis in cattle using ELISA test:

Out of 184 serum samples collected randomly from suspected cattle were examined by ELISA test, there were 24 (13.04%) positive cases in cattle (Table 2). The study show the prevalence of bovine anaplasmosis according to age group, sex and area of infection expressed in tables (3,4,5).

Table (2): Seropositivity rate of Bovine anaplasmosis by using ELISA technique.

No. of examined cattle	No. of positive cases for ELISA test	Infection rate %
184	24	13.04

Table (3): Infection rates according to age of examined animals by using ELISA technique.

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

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

Table (4): Infection rates according to sex of examined animals by using ELISA technique.

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

Similar letters refers to the non-significant differences between sex under $p < 0.05$.

Table (5): Infection rates according to the area of infection by using ELISA technique.

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

Similar letters refers to non-significant differences, while the different letters refers to the significant differences among areas under $p < 0.05$.

Bovine Anaplasmosis according to RFLP-PCR technique:

The use of this technique was aimed to detect *Anaplasma* species which spread in the area of study. PCR analysis of the DNA isolated from blood samples showed that 20 out of the 24 blood samples were *Anaplasma* spp. positive and revealed an expected PCR product of 577 bp in length (Fig. 1).

According to RFLP-PCR, the restriction endonuclease *Bst*1107I which added to *Anaplasma* positive PCR products cut it in to two expected DNA fragments with 509 bp and 68 bp in length respectively and the result was 18 out of 20 blood samples was *Anaplasma marginale* positive (Fig. 2).

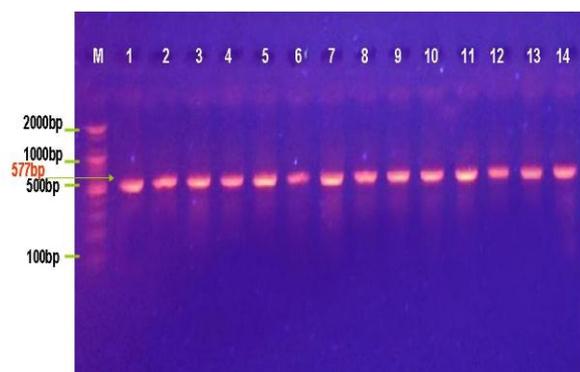
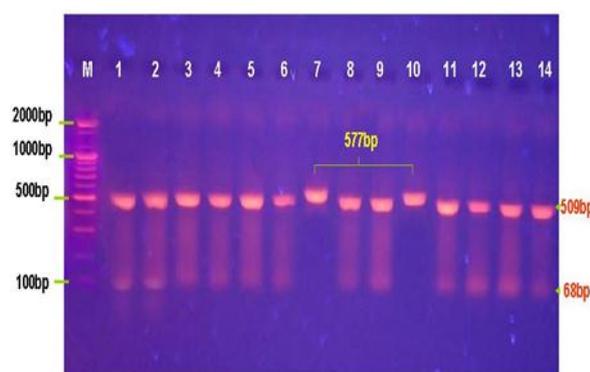
**Fig. 1****Fig. 2**

Fig. 1: Agarose gel electrophoresis image that shown the PCR product analysis of 16S rRNA gene of *Anaplasma* spp. in blood samples. Where M: marker 100bp, lane (1-14) positive blood samples at 577 bp PCR product.

Fig. 2: Agarose gel electrophoresis image that shown the RFLP-PCR product analysis of 577bp of 16S rRNA gene of *Anaplasma* spp. that digestion by restriction endonuclease (*Bst*1107I). Where M: marker 100bp, (lane1,2,3,4,5,6,8,9,11,12,13,&14) positive *Anaplasma marginale* that given cut at 509bp and 68bp, whereas (lane7 and 10) which shown negative for RFLP PCR that still keep at 577bp PCR product .

Discussion

Prevalence of Bovine Anaplasmosis according to ELISA test:

The occurrence of antibodies in the present study against *A. marginale* in cattle from Wassit province is investigated by ELISA technique. Results are demonstrating the infestation of ruminants in the region studied with the hemoparasite (*Anaplasma*), and this result is in agreement with worldwide distribution of anaplasmosis in cattle (15). The results also revealed that the rate of infection in cattle is (13.04 %) and as comparative with other studies done in Iraq, this result is higher than the result recorded by (16) who recorded (9.09%) of cattle in Erbil are infected with Anaplasmosis but lower than the result (30.4) which recorded by (17) in Al-Diwanyia governorate, this difference in results is due to the changes in weather condition and geographic distribution of tick vector between Erbil and AL-Diwanyia or Wassit governorates. The weather is cooler in the north, that lead to lower the distribution of tick vectors which transmitted the disease, while AL-Diwanyia being big agricultural area concentrated animal husbandry especially cows and the warm climate allows the presence of the tick vectors in huge number thus gets larger proportion of infection. Regarding to age wise prevalence, the results of this study revealed that the highest rate of infection in cattle aged over 2 years (16.45 %), while the lowest rate was in calves less than 1 year (8%), statistically there is no significant differences under $p < 0.05$, this result is agreed with many studies applied in this side (17), (18) and (19) whom recorded that the highest prevalence of anaplasmosis in cattle occurs in age 2 years and over. Calves are received temporary immune protection from their mothers by the colostrum, which can prevents Anaplasmosis, this protection lasts about 3 months and in most cases followed by an age resistance which lasts until the animals are about 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 (8, 20). In relation to the sex wise prevalence, the results of the study recorded that females

are more susceptible than male to Anaplasmosis infection in rate 14.14% and 11.7% respectively but the statistically 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 (21). 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, give a good management from owner to the male include good feeding, treatment as ectoparasitic drugs as well as isolated in relatively single and clean yard in order to reach high body weight to sale and gain good pocket money, while female regarded as donors of new calves, all previous causes can lead to decrease exposure of males to parasite of Anaplasmosis (17). The present study showed significant differences in incidence of disease between study districts and area in Wassit 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%, these areas which recorded the high percentage of infection are agricultural areas where available animal husbandry. The wet and warm climate for the tick vectors is the key factor in the transfer of the disease, as these areas are situated near Iran in which many casualties among the herds of cows was recorded and from where possible entry of disease by animals coming into the country through trade or animal movement. The lowest proportion was record 8.33% and 5% in Noamania and Mouafaqia respectively. This may be attributed to the desert nature of these two regions which effect on less animal husbandry, as well as the less presence of the vector tick.

Prevalence of Bovine Anaplasmosis according to RFLP-PCR method:-

Molecular methods, as more sensitive and specific diagnostic tools, have been increasingly used to detect and differentiate *Anaplasma* spp. in carrier animals, it is

recognize even relative amount of DNA of the parasite (22). In the present study DNA extraction was performed on 24 blood samples positive for *Anaplasma* by ELISA, the extracted DNA from blood cells was analyzed by PCR and RFLP-PCR using primers derived from 16S rRNA gene and restriction endonuclease Bst1107I, the restriction endonuclease Bst1107I only recognizes the sequence (GTATAC) in corresponding PCR product of *A. marginale* and cut it (23). According to PCR 20 from 24 samples was 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 bp and 68 bp in length respectively and this confirm diagnosis that cattle in this study was infected with *A. marginale* and the rest 2 negative *A. marginale* result may be infected with *A. centrale* or *A. bovis*. This simple PCR method based on 16S rRNA gene and flowing by RFLP- PCR able to give a rapid discrimination between *A. marginale* and another species of genus *Anaplasma* that infected cattle, the result of this study was agree with the result recorded by (23) 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.

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