DIAGNOSIS OF BOVINE *ANAPLASMA MARGINALE* IN NORTH WESTERN LIBYA USING SEROLOGY AND BLOOD FILM EXAMINATION: A COMPARATIVE STUDY

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ABSTRACT

Anaplasmamarginale (A. marginale) is an obligate intra-erythrocytic rickettsia;it is the cause of anaplasmosis, an important tick-borne disease of cattle. Recovered and vaccinated cattle in endemic areas are apparently normal but remain persistently infected and serve as reservoirs for the parasite.This study intended to detect *A. marginale* in infected and apparently healthy subclinically infected cattle in North Western Libya. During the period extended from March-2006 till September-2007, blood samples and blood smears were collected from totally 119 adult cow (group – I). These cows were raised at some governmental and private farms in Tripoli, Al-Zawiya and Imssallata districts. Blood smears were stained with May-Grunewald-Giemsa stain and examined under Light microscope to detect the presence of intraerythrocytic bacteria. Indirect- ELISA (IELISA) using a 19 KD *A. marginale* recombinant antigen was used to detect serologically positive reactors.During the study period, 20 cases of acute anaplasmosis were diagnosed in these farms (Group-II); where, three cows died and two aborted. The Seroprevalence for *A. marginale* by IELISAwas 64% and 100% in group I and II, respectively. Stained thin blood smears failed to detect infective RBCs in group I, however, variable degrees of parasitaemia were detected in group II.

In conclusion, this study approved that serological test (IELISA) was more reliable

than direct microscopic examination of stained blood smear in detection of chronic persistent anaplasma-infected cows in endemic areas.

INTRODUCTION

Vector-borne diseases impact human and animal health together with its global economy (1). These diseases represent approximately 17% of the burden of all infectious diseases (2). Vector-borne diseases are affecting 80% of the world's cattle population (3). Bovine anaplasmosis is an arthropod- born haemolytic disease of cattle. It occurs in tropical, subtropical countries and in regions with temperate climate (4), and cause a major constrain to cattle production in many countries particularly Africa (5, 6). Warming of weather has expanded the distribution of their vectors, meanwhile, tick-borne diseases are becoming an increasing and serious problem even in Europe (7, 8). Losses due to anaplasmosis are measured through several parameters such as low weight gain, reduction in milk production, abortion, cost of treatment and mortality (6). *Anaplasma marginale* is the most prevalent tick-born pathogen of cattle, with regions of endemicity on the six populated continents (9, 10).

Infection can have a serious effect on previously unexposed adult cattle. Native cattle in endemic areas are exposed to *A. marginale* infection but do not develop overt disease, partly due to existence of enzootic stability, resulting from previous exposure at early age, when there is significant passively acquired and innate immunity (11).

This obligatory intracellular rickettsial bacterium establishes a life-long persistence in infected cattle and serving as a reservoir for continued transmission of the pathogen (12). Persistently infected cattle or carriers have lifelong immunity and resistance to clinical disease on challenge exposure, this is due to emergence of antigenic variants in which new msp2 variants replicate, then controlled by a variant-specific immune response (6). In recovered animals; up to 0.1% of the erythrocytes remain infected, and the direct visualization of pathogens in peripheral blood smear might be extremely challenging (13). Therefore, researcher found that the serological test was the best way to diagnose carriers animals (12). Nevertheless, serological reactions have many drawbacks as cross reactivity with other blood parasites and increase in proportion of false positive results with passing of time (14, 15).To improve serological diagnosis of bovine anaplasmosis, research has focused on the

identification and characterization of *A. marginale* antigens by gene cloning and production of recombinant proteins which may be suitable for more specific and more sensitive serological tests. Among the antigens of interest are five major surface proteins (MSPs) (16, 17, 18 a, 19). Molecular detection of DNA of these intra-erythrocytic bacteria were applied in different endemic areas as real-time PCR, semi nested PCR with high specificity and low cross reactivity (20, 21, 22)

In Libya, bovine anaplasmosis has been reported as endemic disease (23). It was responsible for massive losses in naive exotic breeds of cattle imported to Libya. However, there is a paucity in the studies that dealing with tick- borne disease and its diagnosis. So, this study was designed to diagnose the clinical and subclinical (carriers) cases of anaplasmosis in cattle in North Western Libya using direct blood smear examination and indirect ELISA technique.

MATERIALS AND METHODS

Animals:

Totally, 119 adult Frisian cows raised in governmental (Al-Quea and Angella-3) and private farms (Tripoli: Al-Hashan, Ein-Zara, Wade- Alrabei, Tajora, AL- Zaweya and Imsallata districts). All these farms have history of successive infections with haemoparasitic diseases. This study was extended March-2006 till September-2007. All animals were subjected to complete clinical examination. Blood smears were prepared from these animals (group I), in addition, blood samples (5 ML) were collected from the Jugular vein of each animal in plan and EDTA tubes. Blood samples were collected from twenty cows that revealed clinical acute anaplasmosis during the study period and considered as (group II).

Blood films:

Thin blood films were prepared directly from peripheral blood, which was obtained by the puncture of the ear vein. In addition, blood smears were also prepared from EDTA- jugular blood. All smears were air dried fixed with Methyl alcohol and stained with May-Grunewald-Giemsa stain. All stained blood films were carefully examined under light microscope using oil immersion (X100) to detect parasitized and abnormal RBCs. Number and location of anaplasma inclusions inside infected cells were recorded in addition, the percentages of parasitaemia were also determined.

Serology :

Indirect antibody ELISA kits (IELISA) (Svanova Biotech AB Uppsala, Sweden) for *A. marginale* were used for detection of the seropositive cows. The sera were screened using a 19 KD *A. marginale* recombinant antigen (24). The test was performed according to the instructions of the manufacture. Percent positivity (PP) was calculated as follows: $PP = \frac{\text{Mean OD of sample or negative control} \times 10}{\text{Mean OD for positive control}}$

Where PP <25 is considered as negative result and PP ≥ 25 is considered as positive result. Mean and standard of deviation (SD) of percent positivity (PP) in the two animal groups were calculated and statistically analyzed using the two samples T-Test.

RESULTS

Majority of clinically examined cows in group (I) were emaciated and revealed various degrees of mucous membrane paleness and low milk production. Fever, anorexia and severe depression with pale icteric mucosa were the most prominent signs that appeared in cows of group (II). Meanwhile, three cows of group (II) died and two aborted at late stage of pregnancy. All animals in both groupsrevealed infestation with ticks, in spite of repeated use of acaricides. Anaplasma infected RBCs were detected in all animals of group II, none has been detected in animals of group I (Table-1). Percentage of infected RBCs varies from <1% to about 80 % (Table-2).

Table-1: Results of blood film examination and IELISA in the two animal groups.

Animal group	Case description	Number	Seropositivity (IELISA)	Positivein blood smear	PP Mean ±SD
I	Apparently normal adult cows	119	76(64%)	0	89.0355 ±23.69
II	Cows Infected with acute anaplasmosis	20	20(100%)	20	51.0229 ±23.69

Table-2: Percentage of Parasitaemia and PP for animals in group II.

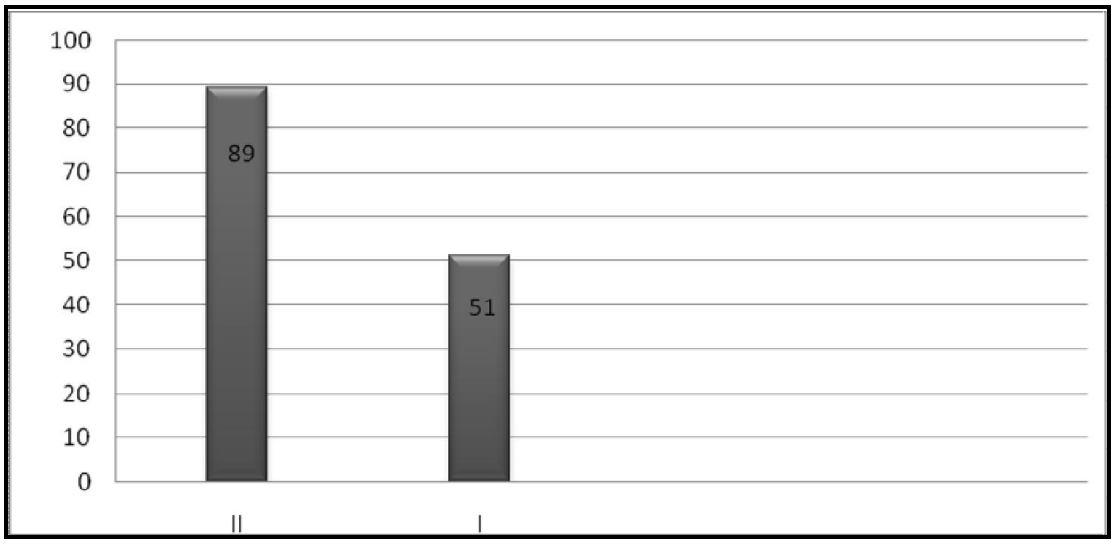
Case no.	Parasitaemia %	PP
1 (18)	30	103.22
2 (19)	2	104.76
3 (20-died)	50	154.16
4 (21)	<1	93.95
5 (26)	< 1	190.32
6 (35-aborted)	50	72.28
7 (36-aborted)	80	60.67
8 (37)	<1	83.59
9 (38)	2	104.63
10 (39)	5	103.62
11 (40)	20	96.57
12 (41-died)	<1	38.65
13 (42)	2	29.26
14 (43)	<1	138.92
15 (44)	<1	128.32
16 (45)	<1	63.82
17 (46)	<1	36.77
18 (48-died)	35	31.34
19 (49)	<1	81.2
20 (50)	10	145.3

Parasitaemia recognized in the stained peripheral blood films were significantly higher than that prepared from jugular blood. All animals in both group even the anaplasma negative animals revealed abnormalities in the morphology of RBCs that are usually associated with bovine haemolytic anaemia, particularly spherocytosis. One to many Anaplasma inclusions was observed in variable size, in one cell. Few of them were tailed. Most of the bacterial inclusions were marginally located, a few were observed in sub-marginal or central locations.

Results of serology (IELISA) (Table1) revealed that 76 animal in group I (64%), and

all animals in group II (100%) reacted positively. Mean of percent positivity (PP) in group II (89.0355 ± 37.568) were significantly (P<0.05) higher than that of group I (51.0229±23.69) (Fig-1) (Table-1).

Fig. 1- Means of pp in group I and II



DISCUSSION

For livestock, bovine anaplasmosis is the most prevalent tick-borne pathogen worldwide (12). In Libya, *A. marginale* was responsible for a severe outbreak among exotic breeds of dairy cattle raised in governmental farms in and around Tripoli at the late nineties and early two thousand (23). The majority of the infected cows died, and those remained alive were weak, unthrifty with low milk productivity.

Through conducting this study it was obvious that applying IELISA is more accurate in diagnosing subclinical cases of anaplasmosis than direct thin blood film examination; however both were equally efficient in detecting acutely infected cows. This finding agreed with previous findings that considered the use of direct blood film examination less reliable than serology for the detection of chronic carriers owing to low parasitaemia (27, 28, and 13). The significant increase in the sensitivity of serology with decrease in the prevalence of piroplasm in the blood has been attributed to stimulation of the immune system that could probably limit the appearance of the piroplasm (29, 30). Nevertheless, elevated titer of antibody cannot eliminate infection, as *A. marginale* has the ability of generating antigenic variants by changing a surface coat composed of numerous proteins, and it is characterized by sequential rickettsemic cycles, in which new MSP2 variants replicate, then controlled by a va

Molecular techniques for the detection of low parasitemia in carrier cattle were applied with high sensitivity and specificity (20, 21, and 22). Anyhow, it appeared that, for underdeveloped and developing countries, microscope and direct blood film examination is still considered the golden standard method for the diagnosis of blood parasites in man and animals(31). Different kinds of ELISA have been conducted with high sensitivity and specificity to determine the prevalence of *A. marginale* in many African and other developing countries (19, 18, 24, 32, 33, 34, 35).

In this study seropositivity in cattle from endemic areas was (64%), this finding in unvaccinated animals is a clear evidence of prior exposure to natural infection and subsequent immunity to it (32).This finding disagreed with the results of others (36), who found 3.4 % seropositivity for *A. marginale* in a study on prevalence of bovine blood parasites in Tripoli districts using ELISA. It is generally accepted that endemic stability to tick-borne diseases exists when the number of sero-positive animals in a herd goes above 81% (38). Based on current results, and beside widely distributed level of antibodies to *A. marginale*, the sampled areas cannot be regarded as having achieved endemic stability with regards to anaplasmosis. As endemic stability refers to a situation where, infectious agents do not cause clinical disease in newly infected hosts under normal circumstances of transmission and infection (35), this finding indicates that cattle in these areas are still susceptible to anaplasma infection.

Clear relation was not detected between outcome of acute anaplasma infection, percent of parasitaemia and antibody titer against anaplasma;cows died from anaplasmosis showed variable degrees of parasitaemia (50%, 35% and < 1%), while the two aborted cows gave the highest parasitaemia (50% and 80%).

Elevated antibody titer (pp154.026) was not protective for the animal (Animal no 3-table-2.

According to previous observations, it seems that antibodies alone were not protective for anaplasmosis. This may be explained by that*A. marginale* clearance is affected by antibodies in combination with cell mediated immunity. It was also suggested that in contrast to the overall titer, antibodies directed specifically against MSPs epitopes are the only antibodies correlating with protection against acute form of the disease. In addition to that scientist proposed that this type of antibody is also required to provide more specificity for erythrophagocytosis and potentiate cell mediated immunity (39, 40, 28, 41). Higher mean PP value in animals of group II is explained by the expected increase in antibody titer during the acute stage that is

usually decreased thereafter with gradual increase in the proportion of false negative results (14). It was also found that increase in optical densities of ELISA is suggestive for the development of active immunity against A. marginale (15).

Our Findings indicated that antibodies against *Anaplasma marginale* infection are widely distributed in cattle raised in the regions included in this study. Positive reactors will continue to act as reservoirs for continued transmission of infection, unless they are treated by a special antibiotic regime supposed to be effective in sterilizing carriers or they are preferably salvaged (42).

تشخيص اصابة الابقار بجرثومة *Anaplasma marginale* في شمال غرب ليبيا باستخدام فحص المسحات الدموية والفحوصات المصليه : دراسة مقارنة

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الخلاصه

تعتبر جرثومة *Anaplasma marginale* الانبلازما من عوامل الركتسيا المهمه المتطفله اجباريا داخل الكريات الدمويه الحمراء ، وهي مسبب لمرض الانبلازموسز الذي يعتبر من الامراض المهمه التي تنتقل عن طريق القراد في الابقار. تبدو الابقار الملقحه و الشافيه من هذا المرض طبيعية ظاهريا، في الاماكن التي يكون فيها المرض متوطنا ، ولكنها تبقى مصابة بالشكل الدائمي للمرض وتكون مصدرا وحاضنا للمسبب المرضي. تعتبر هذه الحيوانات مصدرا للدم الملوث بالجرثومه والمسؤول عن الانتقال الحيوي عن طريق الحشرات او بالطرق الميكانيكيه. تهدف هذه الدراسة إلى الكشف عن جرثومة *A. marginale* في الحالات المصابة سريريا وفي الاصابات المزمنة تحت السريرية و الطبيعية ظاهريا في مناطق شمال غرب ليبيا. تم جمع 119عينة دم وعمل مسحات دموية من الابقار خلال الفترة الممتدة من مارس 2006 وحتى سبتمبر 2007وسميت هذه (بالمجموعة –I). هذه الابقار تمت تربيتها في بعض المزارع الحكوميه والخاصه لتربية الابقار في ضواحي طرابلس، الزاويه و امسلاته في ليبيا. تمصبغ المسحات الدموية بصبغة مي كرينولد-كيمزا وتم فحصها تحت المجهر الضوئي لتشخيص وجود جراثيم الانبلازما في داخل الكريات الدمويه الحمراء. تم اجراء فحص الايلايزا غير المباشر من خلال استعمال المستضد المؤتلف 19 كيلو دالتون لجرثومة*A. marginale* للتحريعن الاصابات السريريه والمزمنه الموجبه للمرض مصليا. خلال فتره الدراسة تم ظهور وتشخيص 20 حاله اصابه بمرض الانبلازما الحادة في هذه الحقول وسميت هذه (بالمجموعه -2)حيث سجل هلاك ثلاثه حيوانات و اجهاض حالتين. اظهر فحص الاليزا نسبة اصابة بجرثومة الانبلازما 64% و 100%، في المجموعه 1و 2 على التوالي. وقد فشل الفحص المباشرللمسحات الدمويه المصبوغه في تشخيص الاصابه من خلال تحديد وجود الجراثيم داخل الكريات الحمراء في المجموعه 1، بينما شخصت درجات متفاوته من طفيلية الدم في المجموعه 2 . لقد انعدمت العلاقه

المباشره بين النسبه المئويه للحالات الموجبه (pp) في فحص الايلايزا غير المباشر ونسبه طفيلية الدم في ابقارالحالات السريريه للمرض . وقد كان معدل ال ppفي مصول ابقار المجموعه 2 اعلى احصائيا ($P<0.05$) من ابقار المجموعه 1.في الخلاصة اثبتت هذه الدراسة بان فحص الايلايزا المصلي غير المباشر(IELISA) اكثر فعالية من الفحص المجهري المباشر لمسحة الدم لتشخيص الاصابة المزمنة المستمرة لجرثومة anaplasma بأبقار المناطق الموبوءة.

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