

# Epidemiological Studies ,Molecular Diagnosis of Anaplasma Marginale in Cattle and Biochemical Changes Associated with it in Kaliobia Governorate

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**Abstract** This investigation is performed on 100 cattle in Kaliobia governorate Egypt aged from 1- 6years severity of illness increase with age , these animals suffered from fever (41 °C) enlargement lymph node and drop in milk yield emaciation in progressive stages , cattle producers first notice the anemic anaplasmosis – infected animal when it becomes weak and lag behind the herd when these animals were subjected to microscopic examination the degree of parasitaemia was recorded as the percentage of infected red blood cells in each blood smear 100 microscopic field were examined .We report the detection of anaplasma marginale by PCR in blood samples obtained from cattle supposed to be infected. The assay employs primers specific for the gene encoding anaplasma marginale specific PCR using primers derived from msp5 gene .The PCR products for 26 positive samples were subjected to sequence ( Labtechnology, Egypt) and BLAST analysis was used for identification of the genomic DNA of these parasites. changes associated with anaplasma marginale in these cattle particular emphasis to the oxidative stress the reduce TAC level may reflect a decrease in antioxidant capacity and CBC change . Blood collected from all animals on EDTA to microscopic examination and PCR to determine type of anaplasma .

**Keywords:** PCR.sequance,anaplasma marginale ,antioxidant, CBC

## 1. Introduction

The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) are obligate intracellular etiological agents of tick borne diseases of mammalian hosts [1], includes the causative agents of anaplasmosis of ruminants. Of these erythrocytic *Anaplasma spp.*, three species, two infecting cattle (*A.marginale* and *A. centrale*) and one in sheep and goats (*A. ovis*) were well-recognized [2]. Acute Anaplasmosis, caused by *A.marginale*, It invades and multiplies in red blood cells. As the disease progresses, infected and even uninfected red blood cells are destroyed mainly in the liver and spleen, resulting in anaemia and even death in severe cases, the number of infected erythrocytes increases drastically and phagocytosis by reticulo-endothelial cells of parasitized erythrocytes lead to development of hemolytic anemia and icterus, Cattle that recover from acute infection become carriers and the parasite can persist most probably for the lifetime in the blood [3]. The disease is characterized by a progressive hemolytic anemia is one of the most important diseases of ruminants worldwide, causing significant economic losses in the tropical and subtropical areas [4]. It seems that the cattle recovered from acute anaplasmosis function as long-term or lifetime carrier [5]. *Anaplasma marginale* is the common pathogen of cattle and is responsible for

substantial economic losses in livestock production in developing countries [6]. Molecular methods, with a high degree of sensitivity and specificity, have been developed to identify *A. marginale* DNA [7,8,9]. Major surface protein 5 (MSP5) is a 19-kDa surface protein highly conserved among different strains of *A. marginale* and *A. ovis* and in *A. centrale* [10]. To test this hypothesis required a determination of the true infection status of cattle within an area where *A. marginale* is endemic. For this purpose, we optimized a specific PCR coupled with sequence analysis to identify *A. marginale msp-5* DNA in blood.

This study was designed and performed to

1. Determine anaplasma marginale in cattle
2. To confirm Anaplasmosis oxidative stress in naturally infected cattle with anaplasma marginale .
3. Biochemical changes associated with anaplasma marginale on CBC changes.

## 2. Material and Methods

In Kaliobia governorate Egypt, anaplasmosis are diagnosed based up on traditional morphological characteristics of Giemsa-stained blood smears, which is not surely applicable for the identifying of the carrier animals. The aim of the present study was the determination of the persistently infected (carrier) cattle in

a region of Kaliobia governorate Egypt with the previous history of acute anaplasmosis. This study was performed on 100 native and crossbreed cattle in Kaliobia governorate in period from February 2011 to August 2011 farms were selected for the study depend on their history of out break of bovine *Anaplasma marginals*. Blood smear sample were collected from jugger vein of hundred nature & crossbred cattle age ranging between 1-9 years. Two thin blood smear from all cattle were prepared immediately after each blood collection microscopic examination were performed for presence of *Anaplasma marginal* in erythrocytes to estimate the percent parasitized erythrocytes as described by [11] and biochemical change associated to anaplasma marginale from antioxidant as (TAC and GSH ) and CBC change (PCV, RBCs, HB, MCV, MCH, MCHC.) The extracted DNA from blood cells were analyzed by *Anaplasma marginale* specific PCR using primers derived from *msp5* gene

### 2.1. DNA Extraction from Blood

The DNA was extracted from each sample by chloroform- isoamyl extraction method (All buffers used according to Sambrook *et al.* (1989). Blood samples typically were obtained as 1ml of whole blood stored in EDTA vacutainer tubes. To each 1ml sample, add 0.8ml 1X SSC buffer, and mix. Centrifuge for 1 minute at 12,000 rpm in a microcentrifuge. Remove 1ml of the supernatant and discard into disinfectant. Add 1ml of 1X SSC buffer, vortex, and centrifuge as above for 1 minute, and remove all of the supernatant. Add 375ul of 0.2M NaOAc to each pellet and vortex briefly. Then add 25 ul of 10% SDS and 5ul of proteinase K (20mg/ml H<sub>2</sub>O) (Sigma P-0390), vortex briefly and incubate for 1 hour at 55 °C. Add 120ul phenol/chloroform/isoamyl alcohol and vortex for 30 seconds. Centrifuge the sample for 2 minutes at 12,000 rpm in a microcentrifuge tube. Carefully remove the aqueous layer to a new 1.5ml microcentrifuge tube, add 1 ml of cold 100% ethanol, mix, and incubate for 15 minutes at -20 °C. Centrifuge for 2 minutes at 12,000 rpm in a microcentrifuge. Decant the supernatant and drain. Add 180ul 10:1 TE buffer, vortex, and incubate at 55 °C for 10 minutes. Add 20ul 2M sodium acetate and mix. Add 500ul of cold 100% ethanol, mix, and centrifuge for 1 minute at 12,000 rpm in a microcentrifuge. Decant the supernatant and rinse the pellet with 1 ml of 80% ethanol.

Centrifuge for 1 minute at 12,000 rpm in a microcentrifuge. Decant the supernatant, and dry the pellet in a Speedy-Vac for 10 minutes (or until dry). Resuspend the pellet by adding 200ul of 10:1 TE buffer. Incubate overnight at 37 °C, vortexing periodically to dissolve the genomic DNA. Store the samples at -20 °C.

### 2.2. Polymerase Chain Reaction (PCR)

One pair of oligonucleotide primers was designated using NCBI website and the contribution of genbank based on the *msp5* gene sequence of *Anaplasma spp* (GenBank accession no. M93392). Primers for the PCR were

Forward primer GTGCTACGATCGCGCCTGCT  
Reverse primer GCCCATGCCACTTCCCACGG

Approximately 100ng DNA was used for the PCR analysis. The PCR was performed in 25 µl total volume including one time PCR buffer, 2.5U Taq Polymerase (Fermentas), 2 µl of each primer( forward & reverse), 200 µM of each dATP, dTTP, dCTP and dGTP (Fermentas) and 1.5mM MgCl<sub>2</sub> in automated Thermocycler (Biorad, USA) with the following program: 5 min incubation at 95 °C to denature double strand DNA, 35cycles of 45s at 94 °C (denaturing step), 1 min at 59 °C (annealing step) and 45s, at 72 °C (extension step). Finally, PCR was completed with the additional extension step (72 °C) for 10 min. The PCR products were analyzed on 1.5% agarose gel in 1X TBE buffer and visualized using ethidium bromide and UV-eluminator.

### 2.3. Sequencing of DNA

The PCR products for 26 positive samples were subjected to sequence ( Labtechnology, Egypt) and BLAST analysis was used for identification of the genomic DNA of these parasites.

## 3. Results

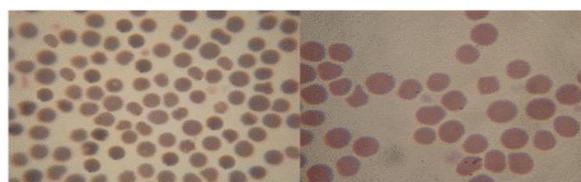
**Table 1:** Showed that significant decrease in RBCs count, PCV% and Hb concentration while **Table 2** Showed that significant difference between control & infected animals (carrier) in GSH and TAC antioxidant. *Anaplasma marginale* considered control.

**Table 1. Mean±SE of CBC in uninfected control cattle and infected cattle with anaplasma marginales**

CBC GROUPS	MCHC	MCH	MCV	HB	RBCS	PCV	MCHC
Control Group I (Negative)	32.97±0.22	19.87±0.13	60.33±0.48	12.83±0.23	6.45±0.11	38.08±0.56	32.97±0.22
Infected Group II (carrier)	33.67±0.16	20.17±0.15	59.93±0.43	10.78±0.14**	5.35±0.08**	32.00±0.36**	33.67±0.16

**Table 2. Mean ±SE of GSH and TAC in uninfected cattle and cattle infected with Anaplasma marginals**

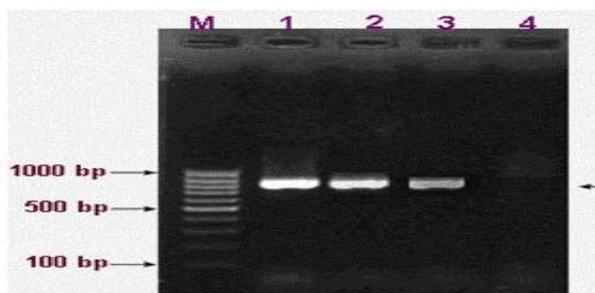
Antioxidants Animals Group	GSH Mmol/l	TAC Mmol/l
Control Group I (negative)	2.83 ±0.10	3.52 ±0.21
Infected cattle Group II (carrier)	1.37 ±0.03***	1.66 ±0.2***



**Figure 1.** showed erythrocytic anaplasma marginals in stained blood film



**Figure 2.** Showed tick infection and showed enlargement in lymph node



**Figure 3.** The PCR product of amplified *A. marginale* msp5 gene separated on 1.5% agarose gel electrophoresis, M: 100bp ladder; lane 1-3 *Anaplasma marginale* msp5, 4:negative control( Without DNA)

Figure 1 showed that Erythrocytic *Anaplasma marginale* in stained blood film with Giemsa stain in cattle while Figure 2 demonstrated cattle infected with tick where *A. marginale* is considered from tick borne disease and prominent enlargement lymph node, Figure 3 Showed that primer pair was tested with DNA from animals suspected to be infected with *A. marginale*. The expected fragment was generated. Results obtained in the PCR assay showed 26 out of 40 samples without clinical signs of infection contact with infected animals and negative microscopic examination (carrier) positive for *A. marginale* of molecular weight 896 base pair. The PCR products for *A. marginale* were subjected to sequence. Blast queries of the resulted sequenced nucleotides indicated the gene identity with msp5 of *A* and the remain samples 14 was negative.

## 4. Discussion

Diagnosis of anaplasma marginal in cattle depend on case history of disease , disease is endemic in Kaliobia and clinical signs appear on animals in acute stage as Anaroxia-fever-diarrhoea &swollen Lymph node-sever anemia decreases apatite. microscopic exam for confirmation in acute cases 10-50% of red blood cells can be infected so they are easy to observe in smear stained with gemisa infected cells and diagnosis 60 infected animals out of 100 animals and the remains animals no clinical and negative microscopic examination .Erythrocytes are the major site of infection in cattle [4]. Hemolytic anemia associated with fever, weight loss, abortion, decreased milk production and in some cases death of the infected cattle [12,13]. Giemsa stained blood smears 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 [14]. All smears were carefully examined for presence of anaplasma margenales in each blood smears 100 microscopic fields were examined per slide

found 10%-50% infected blood cells .Only levels of 106 infected erythrocytes per ml could be detected by Giemsa staining [15]. Cyclical levels of *Rickettsia* in persistently infected cattle fluctuate between 102.5 and 107 infected erythrocytes/ml, with the lowest levels lasting approximately 5-8 days of every 5-6-week cycle. Since the persistently infected cattle can serve as a reservoir for the spread of *A. marginale*,they will be important for both herd health management and movement of animals into and out of the endemic areas [16,17]. The results of this study for diagnosis of *A. marginale* in cattle by PCR analysis revealed that the traditional Giemsa staining method is not applicable for identification diagnosis of persistently infected cattle. Our results showed that *Anaplasma*-like structures could be detected in erythrocytes of 26 sample out of 40 negative blood samples.Due to the difficult differentiation between *Anaplasma* organisms and structures like Heinz bodies, Howell-Jolly bodies or staining artifacts, often seen in Giemsa stained blood smears, DNA from corresponding blood samples were analyzed by PCR. *A. marginale* was detected in 26 out of 40 blood samples using PCR method. msp5 sequence analysis showed high conservation among 26 PCR amplicon sequences from naturally infected cattle. The sequences were over 95% identical to the reference, *A. marginale* [18].The results obtained from blood samples collected from cattle in Kalubia governorate, Egypt showed that the PCR used in this study is more sensitive than detection by light microscopy, which is performed routinely in laboratories in Egypt. Similar results have been reported by [19]. Several serological tests have been established. Unfortunately, because of antigen cross reactivity, these tests do not discriminate between different *Anaplasma* species [20].

Total antioxidant capacity (TAC) is important parameter measured in infected animals it showed significantly lower in Anaplasmosis in contrast to control group. Conclusion the reduce in level of TAC may reflect a decrease also (GSH) is antioxidant capacity of the antioxidant status of tissues be determination of total antioxidant capacity (TAC)&(GSH) [21]. Erythrocytic GSH was significantly reduced in *A. marginale*-infected animals (group II) than healthy animals (group I). Moreover, percentages of parasitemia in infected animals were negatively correlated with erythrocytic GSH in infected calves. .In cattle reduce (TAC) level detected with Anaplasmosis in contrast to healthy control animals in cattle infected with *Anaplasma marginals* . Alteration of oxidative stress indices have been reported in parasitic diseases [22,23]. The antioxidant levels of (RBS) decreases during progression of anemia. Extra-vascular hemolytic anemia is a key feature of anaplas mosis [24,25]. Recently demonstrated parasitemia caused by *A. marginale* augments the mean corpuscular fragility of red cells [26].

CBC of examined animals showed significant change in all parameters as ( PCV ,RBCs, HB , MCV , MCH,MCHC,) were significantly reduce in infected cattle when compared with control negative group .mean values of Hb%, TEC, and PCV% were significantly low in *A. marginale*-infected calves than healthy calves, and significant negative correlation was noted between parasitemia and Hb% and PCV%. ,severe anemia attributable to immune-mediated destruction of non-

parasitized erythrocytes besides parasitized erythrocytes [21]. The results of the present study suggest a possible association between oxidative stress and hemolytic crisis in anaplasma-infected calves. This can be explained by excess free radical generation, occurred due to *A. marginale* infection, than antioxidant capacity [23]. The determination of oxidative stress markers might be helpful to clinicians, and while treating anaplasmosis, incorporation of antioxidants will be helpful for better response in the treatment schedule. Cattle in Kalubia contact to infected animals without clinical signs (suspected for infection) was identified as *A. marginale* infected or uninfected by using the specific PCR. Thus, here we report the use of specific PCR for sensitive and specific amplification of *Anaplasma marginale* DNA from blood samples obtained from cattle. Primers were derived from the gene encoding *Anaplasma marginale* msp5 gene. The resulted PCR-amplified DNA products were analyzed by agarose gel electrophoresis and confirmed by sequencing and blast on the NCBI web. PCR is considered confirmed test for diagnosis of anaplasma marginale in cattle without clinical signs (carrier and early stage of infection) in contact with infected animals in endemic area to make good control and good prognosis in treatment.

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