

## Full Length Research Paper

# Epidemiological studies, molecular diagnosis of anaplasma marginale in cattle and biochemical changes associated with it in Kaliobia Governorate

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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. RBCs count, PCV% and Hb concentration showed significant decrease in infected animals. Blood collected from all animals on EDTA to microscopic examination and PCR to determine type of anaplasma.

**Keywords:** PCR.Sequence, Anaplasma marginale, antioxidant and CBC

## INTRODUCTION

The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) are obligate intracellular etiological agents of tick borne diseases of mammalian hosts (Dumler et al., 2001), includes the causative agents of anaplasmosis of

ruminants. Of this erythrocytic *Anaplasma spp.*, three species, two infecting cattle (*A. marginale* and *A. centrale*) and one in sheep and goats (*A. ovis*) were well-recognized (Lestoquard, 1924; Theiler, 1910). 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

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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 (Vahid et al., 2009). 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 (Kocan et al., 2003). Hemolytic anemia associated with fever, weight loss, abortion, decreased milk production and in some cases death of the infected cattle (Palmer et.al 1989 and Ahmed El sawlhy 1999). 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 (Carelli et al., 2007). It seems that the cattle recovered from acute anaplasmosis function as long-term or lifetime carrier (Eriks et al., 1993). 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 (Bradway et al., 2001; Tarek et al., 2009). Therefore, several serological tests have been established. Unfortunately, because of antigen cross reactivity, these tests do not discriminate between different *Anaplasma* species (Jimenez et al., 2008). *Anaplasma marginale* is the common pathogen of cattle and is responsible for substantial economic losses in livestock production in developing countries. (Dreher et.al. 2005). Molecular methods, with a high degree of sensitivity and specificity, have been developed to identify *A. marginale* DNA (Carelli et al., 2007; Bekker et al., 2002; Molad et al., 2006). 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* (Ndung'u et al., 1995). 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. 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 site.

This study was designed and performed to

- 1- Determine *A. marginale* in cattle.
- 2- To confirm Anaplasmosis oxidative stress in naturally infected cattle with *A. marginale*.
- 3- Haematological changes associated with *A. marginale* (CBC).

## 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 crossbreeds 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 and 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 *A. marginale* in erythrocytes to estimate the percent parasitized erythrocytes as described by (Coetzee et al., 2005). Blood was collected from jugular vein from each animal in centrifuge tubes using disodium salt of ethylene diamine tetraacetic acid (Na<sub>2</sub>-EDTA) as anticoagulant. Total hemoglobin (Hb grams per deciliter), Packed cell volume (PCV%) and total erythrocytic count (TEC) were analyzed for hematology. Hemoglobin was estimated according to Van Kampen and Zijlsta (1961). Total erythrocytic count and PCV were estimated according to Feldmen et al, (2000). Red cell indices were calculated from the measured PCV, Hb and RBCs count according to Feldmen et al, (2000). The activity of total antioxidant capacity (TAC) was carried out using commercially available test kits, according to the method described by Koracevic et al., (2001). And the activity of reduced glutathione (R.GSH) was determined in erythrocyte hemolysate according to the method described by Beutler et al, (1963). The extracted DNA from blood cells was analyzed by *Anaplasma marginale* specific PCR using primers derived from *msp5* gene.

**Table 1.** Mean  $\pm$  SE of CBC in uninfected control cattle and infected cattle with *Anaplasma marginale*

| CBC GROUPS                  | PCV                      | RBCS                    | HB                       | MCV                    | MCH                    | MCHC                   |
|-----------------------------|--------------------------|-------------------------|--------------------------|------------------------|------------------------|------------------------|
| Control Group 1 (Negative)  | 38.08<br>$\pm$<br>0.56   | 6.45<br>$\pm$<br>0.11   | 12.83<br>$\pm$<br>0.23   | 60.33<br>$\pm$<br>0.48 | 19.87<br>$\pm$<br>0.13 | 32.97<br>$\pm$<br>0.22 |
| Infected Group II (carrier) | 32.00<br>$\pm$<br>0.36** | 5.35<br>$\pm$<br>0.08** | 10.78<br>$\pm$<br>0.14** | 59.93<br>$\pm$<br>0.43 | 20.17<br>$\pm$<br>0.15 | 33.67<br>$\pm$<br>0.16 |

### 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 1 ml of whole blood stored in EDTA vacutainer tubes. To each 1 ml sample, add 0.8 ml 1X SSC buffer, and mix. Centrifuge for 1 minute at 12,000 rpm in a microcentrifuge. Remove 1 ml of the supernatant and discard into disinfectant. Add 1 ml of 1X SSC buffer, vortex, and centrifuge as above for 1 minute, and remove all of the supernatant. Add 375  $\mu$ l of 0.2M NaOAc to each pellet and vortex briefly. Then add 25  $\mu$ l of 10% SDS and 5  $\mu$ l of proteinase K (20 mg/ml H<sub>2</sub>O) (Sigma P-0390), vortex briefly and incubate for 1 hour at 55°C. Add 120  $\mu$ l 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 1.5 ml 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 180  $\mu$ l 10:1 TE buffer, vortex, and incubate at 55°C for 10 minutes. Add 20  $\mu$ l 2 M sodium acetate and mix. Add 500  $\mu$ l 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 200  $\mu$ l of 10:1 TE buffer. Incubate overnight at 37°C, vortexing periodically to dissolve the genomic DNA. Store the samples at -20°C.

### Polymerase chain reaction (PCR)

One pair of oligonucleotide primers was designated using NCBI website and the contribution of genebank based on

the msp5 gene sequence of *Anaplasma* spp (Gen Bank accession no. M93392). Primers for the PCR were

Forward primer GTGCTACGATCGCGCCTGCT

Reverse primer GCCCATGCCACTTCCCACGG

Approximately 100 ng DNA was used for the PCR analysis. The PCR was performed in 25  $\mu$ l total volume including one time PCR buffer, 2.5 U Taq Polymerase (Fermentas), 2  $\mu$ l of each primer (forward and reverse), 200  $\mu$ M of each dATP, dTTP, dCTP and dGTP (Fermentas) and 1.5 mM MgCl<sub>2</sub> in automated Thermocycler (Biorad, USA) with the following program: 5 min incubation at 95°C to denature double strand DNA, 35 cycles of 45 s at 94°C (denaturing step), 1 min at 59°C (annealing step) and 45 s, 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-illumination.

### 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.

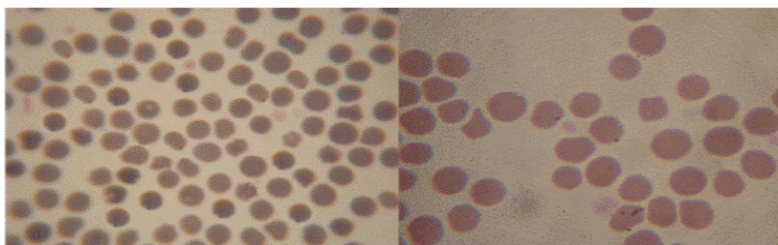
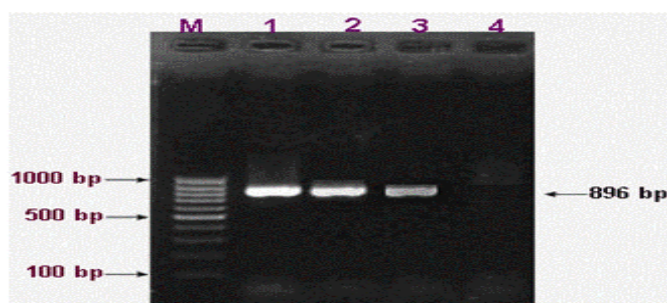
## RESULTS

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

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

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

| Antioxidants<br>Animals Group      | GSH Mmol/l            | TAC Mmol/l           |
|------------------------------------|-----------------------|----------------------|
|                                    |                       |                      |
| Control Group I (negative)         | $2.83 \pm 0.10$       | $3.52 \pm 0.21$      |
| Infected cattle Group II (carrier) | $1.37 \pm 0.03^{***}$ | $1.66 \pm 0.2^{***}$ |

**Figure 1.** showed erythrocytic anaplasma marginals in stained blood film**Figure 2.** Showed tick infection      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)

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.

## 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 and swollen Lymph node-sever anemia decreases apatite. microscopic examination for confirmation in acute cases 10-50% of red blood cells can be infected so they are easy to observe in smear stained with Giemsa infected cells and diagnosis 60 infected animals out of 100 animals and the remains animals no clinical and negative microscopic examination this results agreed with (Kocan Km et.al. 2003) who said that erythrocytes are the major site of infection in cattle. All smears were carefully examined for presence of *A. marginale* in each blood smears 100 microscopic fields were examined per slide found 10%-50% infected blood cells this result agreed with (Gale et al. 1996) showed that only levels of 106 infected erythrocytes per ml could be detected by Giemsa staining.

The result 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 and diagnosis of persistently infected cattle (carrier) with no clinical signs and apparently healthy in contact with apparently diseased animals. Our results showed that *A. marginale* could be detected in erythrocytes of 26 samples out of 40 negative microscopic examination 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 this results came agreement with (Toriainet et.al.1998). The sequences were over 95% identical to the reference, *A. marginale*. 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 Hofmann *et al* (2004).

Total antioxidant capacity (TAC) and reduced glutathione (R.GSH) are important parameteres measured in infected animals which reported positive by PCR they showed significantly lower in *A. marginale* in contrast to control group, erythrocytic GSH was significantly reduced in *A. marginale*-infected animals (group I) than healthy animals (group II). In cattle reduce

(TAC) level detected with Anaplasmosis in contrast to healthy control animals in cattle infected with *A. marginale*. Alteration of oxidative stress indices have been reported in parasitic diseases this result came agreement with (Dede et al. 2002, Pabon et al. 2003 and (Ujjwal et al., 2011) who said that the antioxidant levels of (RBS) decreases during progression of anemia so determination of (TAC) and (GSH) reflect decrease antioxidant capacity of the antioxidant status of tissues ,in other hand (Latimer et al., 2003 Murat Guzel et al., 2008) reported that Extra-vascular hemolytic anemia is a key feature of anaplasmosis. Recently, (Nazifi et al., 2008) demonstrated parasitemia caused by *A. marginale* augments the mean corpuscular fragility of red cells.

PCV%, TECand Hb% concentration of examined animals showed significant decrease in infected cattle when compared with control group this results came agreement with (Ujjwal et al., 2011) who concluded that mean values of Hb%, TEC, and PCV% were significantly low in *A. marginale*-infected animals than healthy animals and explain the cause of Severe anemia attributable to immune-mediated destruction of non-parasitized erythrocytes besides parasitized erythrocytes. The results of the present study suggest a possible association between oxidative stress and hemolytic crisis in anaplasma-infected animals this can be explained by (Pabon et al., 2003) who said that excess free radical generation, occurred due to *A. marginale* infection, than antioxidant capacity.

## CONCLUSION

1-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.

2- 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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