

Biochemical Changes Associated with Anaplasma Infection in Cattle

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Abstract: This investigation is performed on 100 cows aged from (1-6) years. These animals suffered from the major symptoms of anaplasmosis as fever (41°C) enlargement lymph node and drop in milk yield, emaciation and anemia. Blood samples were collected from all animals on EDTA for microscopic examination and PCR for diagnosis of anaplasmosis. *Anaplasma marginale* (*A. marginale*) was detected by PCR in blood samples obtained from cattle supposed to be infected. The assay employs primers specific for the gene encoding *A. marginale* specific primers derived from major surface protein 5 (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 *A. marginale* in these cattle particular emphasis to the oxidative stress the reduce TAC level may reflect a decrease in antioxidant capacity and CBC change.

Key words: PCR • Anaplasma • Antioxidant • CBC

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. As the disease progresses, infected and even uninfected red blood cells are destroyed mainly in the liver and spleen, resulting in anemia 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 [2]. The disease is characterized by a progressive hemolytic anemia [3]. Hamolytic anemia associated with fever, weight loss, abortion, decreased milk production and in some cases death of infested cattle[4] and [5]. It seems that the cattle recovered from acute anaplasmosis function as long-term or lifetime carrier[6]. Since the

persistently infested cattle can serve as a reservoir for the spread of *A. marginale*, they will be movement of animals into and out of the endemic areas [7] and [8]. Therefore, several serological tests have been established. *A. marginale* is the common pathogen of cattle and is responsible for substantial economic losses in livestock production in developing countries [9]. Molecular methods, with a high degree of sensitivity and specificity, have been developed to identify *A. marginale* DNA [10]. msp5 is a 19-kDa surface protein highly conserved among different strains of *A. marginale*, *A. ovis* and *A. centrale* [11]. 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.

Oxidative stress may result from an imbalance between reactive oxygen species (ROS) and antioxidants levels [12]. It is well known that ROS are produced by several pathological conditions and cause cellular damages such as lipid peroxidation and protein oxidation.

The biological oxidative effects of free radicals on lipids and proteins are controlled by a spectrum of antioxidants [13]. The antioxidant status of tissues can be described by the analysis of single components in the defense systems against ROS, as well as by the determination of total antioxidant capacity (TAC). In contrary, the TAC measurement does not represent the sum of activities of antioxidants; it could be used for clinical diagnosis, as it is an easy and less time-consuming procedure [14].

This study was designed and performed to detect *A. marginale* by PCR and to determine oxidative stress and biochemical changes associated with *A. marginale* in naturally infected cattle.

MATERIAL AND METHODS

Animals: This study was performed on 100 native and crossbreed cattle farms in period from February 2011 to August 2011 were selected for the study depends on their history of outbreak of bovine anaplasmosis.

Microscopy Detection Method: Two thin blood smears from all cattle were prepared immediately after each blood collection. Microscopic examination were performed for presence of *A. marginale* in erythrocytes to estimate the percentage of parasitized erythrocytes as described by Coetzee *et al.* (2005) [15]. Blood was collected from jugular vein of each infected animal in centrifuge tubes using disodium salt of ethylene diamine tetra-acetic acid (Na₂-EDTA) as anticoagulant. The degree of parasitaemia was recorded as the percentage of infected red blood cells in 100 microscopic field in each blood smear.

Haematological investigations Hemoglobin was estimated according to Van Kampen and Zijlsta [16]. Total erythrocytic count and PCV were estimated according to Feldmen *et al.* [17]. The activity of total antioxidant capacity (TAC) according to the method described by Koracevic *et al.* [18] and the activity of reduced glutathione (GSH) was determined in erythrocyte hemolysate according to the method described by Beutler *et al.* [19].

Molecular Detection of *A. marginale*: The DNA from 40 samples (suspected positive) was extracted using chloroform-isoamyl extraction method according to Sambrook *et al.* [20]. The extracted DNA from blood cells was analyzed by *A. marginale* specific PCR using primers derived from *msp5* gene..

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 designated as follows:

Forward primer GTGCTACGATCGCGCCTGCT
Reverse primer GCCCATGCCACTTCCCCACGG

Approximately 100ng DNA was used for the PCR analysis. The PCR was performed in 25 μ l total volume including one time PCR buffer, 2.5U Taq Polymerase (Fermentas), 2 μ l of each primer (forward and reverse), 200 μ M of each dATP, dTTP, dCTP and dGTP (Fermentas) and 1.5mM MgCl₂ 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.

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

The data summarized in Table (1) showed a significant decrease in RBCs count, PCV% and Hb concentration of infected cattle with *A. marginale* in comparison with normal cattle. The results obtained in Table (2) showed a significant decrease in the serum level of GSH and TAC in cattle infected with *A. marginale* in comparison with normal cattle

Figure two showed that primer pair was tested with DNA from animals suspected to be infested with *A. marginale*. The expected fragment (896 bp) was generated. Results obtained in the PCR assay showed 26 out of 40 samples without clinical signs of infection and with negative microscopic examination (carrier) were positive for *A. marginale*. The PCR products for *A. marginale* were subjected to sequenc. Blast queries of the resulted sequenced nucleotides indicated the gene identify with *msp5*.

Table 1: Haematological profile in non-infected and infected cattle with *A. marginale* detected by different methods

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

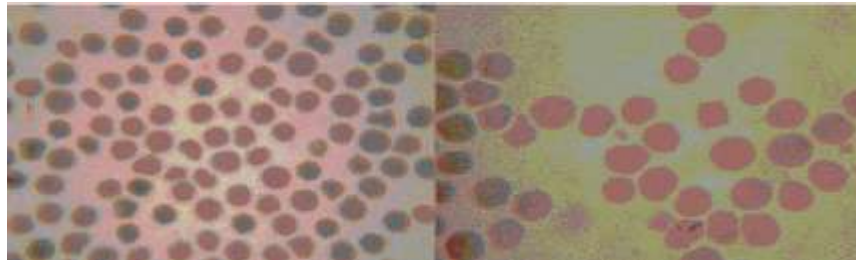


Fig. 1: Cattle erythrocytes infected with *A. marginale* in stained blood film with Giemsa stain.

Table 2: GSH and TAC antioxidants profile in normal and infected cattle with *A. marginale*

Animals Group	GSH Mmol/l	TAC Mmol/l
normal Group I	2.83 ± 0.10	3.52 ± 0.21
Infected cattle Group II	1.37 ± 0.03***	1.66 ± 0.2***

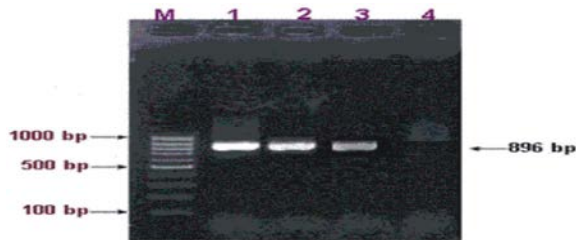


Fig. 2: The PCR product of amplified *A. marginale* msp5 gene separated on 1.5% agarose gel electrophoresis, M: 100bp ladder; lane 1-3 *A. marginale* positive samples, 4: negative control (Without DNA)

DISCUSSION

In the present study, diagnosis of anaplasmosis in cattle depends on case history of disease and microscopic examination for confirmation in acute cases (10-15 % of red blood cells were infected) so they are easy to observe in smear stained with Giemsa. Out of 100 animals 60 were positive using microscopic examination. These results agreed with Kocan Km *et al.* [3] who stated that erythrocytes are carefully examined for presence of *A. marginale* in each blood smears 100 microscopic fields were examined per slide found 10-50% infected blood cells. Gale *et al.* [21] showed that, only levels of 106 infected erythrocytes per ml could be detected by Giemsa staining.

Traditional Gemisa staining method is not applicable for identification and diagnosis of persistently infected cattle (carrier) with no signs and apparently healthy in contact with diseased animals Diagnosis of anaplasmosis in cattle is difficult due to the difficult differentiation between Anaplasma organisms, structures like Heinz bodies, Howell-Jolly bodies or staining artifacts, often seen in Giemsa stained blood smears. So, DNA from corresponding blood samples were analysed by PCR.. Our results showed that, *A. marginale* could be detected in erythrocytes of 26, out of 40 blood samples by using PCR method. Sequences analysis of the PCR products showed high conversation among 26 PCR amplicon sequences from naturally infected cattle this results were in an agreement with Toriainet *et al.* [22]. The from the previous data, we concluded that PCR is more sensitive than detection by traditional light microscopy.

Total antioxidant capacity (TAC) and reduced glutathione (GSH) are important parameters measured in infected animals which reported positive by PCR. Our result showing significant decrease of TAC level in cattle infected with *A. marginale* when compared with control group this result is agreed with Rezai *et al.* [23] who reported that, antioxidant level of RBCs decreased during the progression anaemia. this may suggest the alternations in ant oxidative and oxidative balance due to oxidative stress and ROS generation in the course of anaplasma. furthermore, this alteration may be due to decrease in the level of enzymatic and non enzymatic antioxidants which are the component of antioxidant-defence system Serdar *et al.* [13]. In the present study revealed a significant decrease in GSH level in cattle infected with *A. marginale* this data are in harmony with Silva *et al.* [24] who mentioned reduction in GSH level which may be resulted from the activity of GPX in

reducing lipid hydroperoxides to stable non-radical lipid alcohols, utilizing GSH as the source of reducing equivalents alternatively, GSH concentration may have been reduced by the direct utilization of RBCs GSH as an antioxidant due to hydrolysis of RBCs. Moreover, glutathione is presumed to be an important endogenous defense against the peroxidative destruction of cellular membrane. GSH can act either to detoxify activated oxygen species such as H₂O₂ or to reduce lipid peroxides themselves Frerman and Crapo[25]. Oxidative stress may result from either an increase in ROS production or a decrease in the activity of antioxidant enzymes or a decrease in the concentration of non enzymatic antioxidants such as GSH Chaunhan *et al.* [26]. GSH may convert H₂O₂ to H₂O by PGX and produce GSSG. GSSG can be converted back to GSH by glutathione reductase Resende *et al.* [27].

Alteration of oxidative stress indices have been reported in parasitic diseases this result came agreement with Pabon *et al.* [28] who reported 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. Latimer *et al.* [29] Murat Guzel *et al.* [30] reported that, extra-vascular hemolytic anemia is a key feature of anaplasmosis.

PCV%, TEC and Hb% concentration of examined animals showed significant decrease in infected cattle when compared with control group this results are agreement with Ujjwal *et al.* [31] 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 severanaemia 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.* [28] who said that, excess free radical generation, occurred due to *A. marginale* infection, than antioxidant capacity.

CONCLUSION

Oxidative stress markers which indicated by reduced level of TAC act a reference to decrease host antioxidant capacity.

Using PCR test for diagnosis of *Anaplasma marginale* in cattle help for isolate the infected from carrier diseased cattle in endemic area to make good control.

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