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 governmorate Egypt aged from 1-6 years 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 wear 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; sequence, 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 reticuloendothelial 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 Kalobiya governorate Egypt with the previous history of acute anaplasmosis. This study was performed on 100 native and crossbred cattle in Kalobiya governrate 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 erythroctes as described by [11] and biochemical change associated to anaplasma marginals 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 marginal 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 H2O) (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 genebank based on the msp5 gene sequence of Anaplasma spp (GenBank accession no. M93392). Primers for the PCR were

Forward primer: GTGCTACGATCGCGCTGCT
Reverse primer: GCCCATGCACCTCGCTACGG

Approximately 100ng DNA was used for the PCR analysis. The PCR was performed in 25µl total volume including one time PCR buffer, 2.5U Taq Polymerase (Fermentas), 2µl of each primer (forward & reverse), 200µM 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.

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 marginal considered control.

<table>
<thead>
<tr>
<th>CBC GROUPS</th>
<th>MCHC</th>
<th>MCH</th>
<th>MCV</th>
<th>HB</th>
<th>RBCS</th>
<th>PCV</th>
<th>MCHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group 1 (Negative)</td>
<td>32.97±0.22</td>
<td>19.87±0.13</td>
<td>60.33±0.48</td>
<td>12.83±0.23</td>
<td>6.45±0.11</td>
<td>38.08±0.56</td>
<td>32.97±0.22</td>
</tr>
<tr>
<td>Infected Group II (carrier)</td>
<td>33.67±0.16</td>
<td>20.17±0.15</td>
<td>59.93±0.43</td>
<td>10.78±0.14**</td>
<td>5.35±0.08**</td>
<td>32.00±0.36**</td>
<td>33.67±0.16</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Animals Group</th>
<th>GSH Mmol/l</th>
<th>TAC Mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group I (negative)</td>
<td>2.83 ±0.10</td>
<td>3.52 ±0.21</td>
<td></td>
</tr>
<tr>
<td>Infected cattle Group II (carrier)</td>
<td>1.37 ±0.03***</td>
<td>1.66 ±0.2***</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. showed erythrocytic anaplasma marginal in stained blood film.
Diagnosis of anaplasma marginal in cattle depend on case history of disease, disease is endemic in Kalobia and clinical signs appear on animals in acute stage as Anoaxia-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 gemsa 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 s mears 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]. Cyclic levels of Rickettsia in persistently infected cattle fluctuate between 102.5 and 107 infected erythrocytes/ml, with the lowest level 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 oxidative stress indices have been reported in parasitic diseases [22,23]. The antioxidative stress indices have been reported in parasitic diseases [22,23]. The antioxidative stress indices have been reported in parasitic diseases [22,23]. The antioxidative stress indices have been reported in parasitic diseases [22,23]. The antioxidative stress indices have been reported in parasitic diseases [22,23]. The antioxidative stress indices have been reported in parasitic diseases [22,23]. The antioxidative stress indices have been reported in parasitic diseases [22,23]. The antioxidative stress indices have been reported in parasitic diseases [22,23]. The antioxidative stress indices have been reported in parasitic diseases [22,23]. The antioxidative stress indices have been reported in parasitic diseases [22,23].

Discussion

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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References