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Significance of clinical variables and selected biochemical markers in predicting the outcome of bovine anaplasmosis

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ABSTRACT: The present study was aimed at evaluating the usefulness of selected inflammatory and oxidative stress markers in predicting the clinical outcome of cattle infected with *Anaplasma (A). marginale*. The study population consisted of 39 cattle naturally infected with *A. marginale.* The presumptive diagnosis of this infection was initially achieved on the basis of case history, microscopy and clinical examination findings, and confirmed using *A. marginale*-specific PCR assays. The diseased cattle were categorised according to the clinical outcome into survivors (n = 26) and non-survivors (n = 13). For comparison, ten clinically healthy cattle were randomly selected and served as controls. Blood was drawn from all examined animals to measure the respective levels of selected cytokines, acute phase proteins, oxidative stress markers, and antioxidant enzyme levels. We found that the clinical examination alone was not conclusive and should be used in conjunction with other diagnostic methods. Nonetheless, the non-surviving animals showed anorexia, frequent coughing, dyspnoea, bloody faeces, recumbency, pale or icteric mucous membranes, and haemoglobinuria. Biochemically, tumour necrosis factor alpha, interleukin (IL)-1β, IL-6, serum amyloid A, fibrinogen, malondialdehye, superoxide dismutase, and catalase levels were significantly higher in diseased cattle compared with controls, and were higher in non-survivors than survivors ($P < 0.05$). In contrast, reduced glutathione (G-SH) was significantly lower in non-surviving cattle than survivors and controls. Interestingly, a significant correlation was found between parasitaemia of the diseased cattle and most of the measured biochemical variables, with IL-1β and G-SH showing the highest correlation. Our findings clearly demonstrate that *A. marginale* infection is associated with marked inflammatory and oxidative stress responses, which are higher in non-surviving cattle compared with survivors. The overall degree of cytokine and anti-oxidative disruption may have an important prognostic value for the disease outcome.

Keywords: *Anaplasma marginale*; cytokine profile; oxidative stress; prognosis; cattle

Anaplasmosis is considered one of the most prevalent tick-borne diseases that continue to constrain cattle productivity worldwide (Kocan et al. 2010). It is caused by a rickettsial haemoparasite, *Anaplasma (A.) marginale.* The infection has a worldwide distribution and is transmitted biologically by several genera of ixodid ticks. In cattle, *A. marginale* is highly pathogenic and causes a disease characterised by progressive anaemia and icterus, while *A. centrale* causes mild infections (Dumler et al. 2001). The recovery from acute *A. marginale* infections in cattle results in a chronic carrier state characterised by long-term persistent infections that are microscopically undetectable (Eriks et al. 1993). It is well known that the *A. marginale* parasite begins its course by invading and multiplying within red blood cells of the host, which results in anaemia; however, the exact mechanism remains to be clarified (Kocan et al. 2010).

Systemic inflammatory responses play an important role in several pathophysiological processes, such as infections or trauma, and characterised by

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the endocrine release of different cytokines normally confined to paracrine regulation of a local inflammatory response (Koj 1997). The circulating cytokines stimulate the release of acute phase proteins (APPs) from the liver; these play an important role in the innate immune response (Eckersall 2000). Although the precise role of APPs is not well established, their blood levels may be correlated with the severity of inflammation or infection and therefore could serve as helpful biochemical indicators of the inflammatory response (El-Ashker et al. 2014; El-Sebaei et al. 2014). The exact type of APPs and the time course of their release are variable among different species depending on the causative agent and the underlying inflammatory process (Feldman et al. 2000).

To date, little information is available regarding the oxidative stress status and the host inflammatory responses during natural infections with bovine anaplasmosis. A recent report has shown that *A. marginale* infection in cattle is associated with an acute phase response with elevated serum sialic acid levels (Nazifi et al. 2012). Yet, little is known about the molecular characterisation of *A. marginale* infection in cattle in Egypt, and currently, relatively few reports are available which describe this clinical condition (Younis et al. 2009; Salm et al. 2011; Abdel Hamid et al. 2014; El-Ashker et al. 2015). Therefore, the present study was designed to evaluate the acute phase response in cattle naturally infected with *A. marginale* and to determine the significance of clinical variables as well as selected biochemical markers in predicting the outcome of this disease.

MATERIAL AND METHODS

Animal description and selection criteria. The study population consisted of 39 cattle naturally infected with *A. marginale*, aged between two to five years, from three herds located in Dakahlia Governorate, Egypt during the summer seasons of 2013 and 2014. The complaint of the local veterinarians was sudden attack of high fever, anorexia, pale mucous membranes, jaundice, accelerated heart and respiratory rates, constipation in some cases and diarrhoea in others. Sudden deaths were also reported. *A. marginale* infection was initially diagnosed on the basis of case history, microscopy and clinical examination findings, and then confirmed using species-specific PCR assays. Diseased cattle were included in this study if they fulfilled the following criteria: adult cattle (more than two years old) with *A. marginale* infection on the basis of case history, clinical examination findings (high fever > 40 °C, anorexia, abnormal mucous membrane colour, tachycardia, tachypnoea, and heavy tick infestation in some animals), and laboratory findings including (1) microscopic examination of Giemsa-stained blood film; (2) anaemia detected by estimating the packed cell volume (PCV%), erythrocyte count, and haemoglobin values and (3) PCR assays using specific primers for *A. marginale.* The exclusion criteria included: (1) cattle less to two years old; (2) cattle infected with babesiosis and theileriosis that were detected by microscopy and/or PCR assays, or animal harbouring mixed infections with *A. marginale*; (3) cattle received medical treatment prior to blood sampling. Based on the clinical outcome, the diseased cattle were categorised into survivors (n = 26) and non-survivors (n = 13). For comparison, ten clinically healthy cattle were randomly selected and served as a control group.

Clinical examination. All diseased cattle as well as controls were subjected to thorough clinical examination. In particular, general demeanour, physical condition, appetite, act of urination, colour of urine, manner of defecation, rectal temperature, heart and respiratory rates, colour of visible mucous membranes, and superficially located lymph nodes were clinically examined and the findings were reported. The lungs were thoroughly examined for abnormalities. All animals were also examined for the presence of external parasites.

Sampling protocol. Blood was collected from the jugular vein and was divided into three tubes, a plain tube, a tube with EDTA, and a tube with heparin. Blood with EDTA was used for DNA extraction, PCR assays, and haematological examinations. Blood in the plain tube was left for 15 min at room temperature to coagulate and was then centrifuged at 3000 × g for 15 min for separation of blood sera. Only non-haemolysed sera were harvested and were kept frozen at –20 °C until required. The serum samples were used for measuring the following cytokines using commercially available bovine species-specific ELISA kits (MyBioSource Inc., San Diego, USA): tumour necrosis factor alpha (TNF-α) (cat No. MBS283323), interleukin (IL)-1β (cat No. MBS2000207), IL-6 (cat No. MBS564191). The levels of these cytokines

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were measured in undiluted serum according to the manufacturer's instructions. The plates were read at 450 nm and a correction wave length of 550 nm using an automated microplate ELISA reader (Bio TEC, ELX800G, USA). Values were expressed in picograms per millilitre (pg/ml). Samples were run in duplicate for each of the examined cytokines. The heparinised blood was rapidly centrifuged at 3000 × g for 10 min for separation of blood plasma. Commercially available ELISA kits supplied by MyBioSource Inc. were used for estimating serum amyloid A (SAA) (cat No. MBS2024318) and fibrinogen (Fb) (cat No. MBS281998) in collected plasma samples according to the manufacturer's instructions, and an automated microplate ELISA reader (Bio TEC, ELX800G, USA). The selected oxidative stress and anti-oxidant markers including malondialdehyde (MDA; cat No. MD 25 29), reduced glutathione (G-SH; cat No. GR 25 11), catalase (CAT: cat No. CA 25 17), and superoxide dismutase (SOD; cat No. SD 25 21) were measured using commercially available kits (Bio-diagnostic, Egypt) according to manufacturer's instructions. The above-mentioned cytokines and APPs were measured only in the samples collected from animals that were positive for *A. marginale* by PCR assays as well as those from controls.

Ear vein blood samples were also collected and used for blood smear preparation as previously described by El-Ashker et al. (2015). After examining more than 50 microscopic fields of blood film, the parasitaemia is quantified and expressed as the percentage of infected erythrocytes. However, negative blood films did not necessarily imply the absence of infection. Therefore, the presence or absence of infection was confirmed by PCR.

DNA extraction and PCR amplification. Total DNA was extracted from the whole blood of suspected cases using the DNA Isolation Kit for Cells and Tissues (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. Positive control samples representing *Theileria annulata*, *Babesia bovis* and *A. marginale* were obtained from clinically diagnosed cases at the Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt. DNA concentration was measured using a NanoDropTM ND-1000 Spectrophotometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany), and DNA quality was assessed using 2% agarose gel electrophoresis. All extracted DNA samples were stored at –20 °C until required.

For PCR assays of *A. marginale*, one set of specific primers was used to amplify an approximately 95 bp fragment as previously described by Carelli et al. (2007). The sequences of the primers are as follows: forward 5'-TTGGCAAGGCAGCAGCTT-3', and reverse 5'-TTCCGCGAGCATGTGCAT-3'**.** PCR assays were performed in a total reaction volume of 50 µl using PCR Master Mix (Jena Bio-science GmbH) and were run on a Master cycler personal (Eppendorf, Hamburg, Germany) with the following conditions: an initial denaturation at 96 °C for 1 min, followed by 35 cycles of denaturation at 96 °C for 15 s, annealing at 53 °C for 1 min, and extension at 72 °C for 20 s. The final extension was performed at 72 °C for 5 min. Positive and negative controls were always included in each reaction.

For amplification of *Theileria* and *Babesia* species, one set of primers was used as previously described (Gubbels et al. 1999). The primer sequences were as follows: forward 5'-GACACAGGGAGGTAGTGACAAG-3', reverse 5'-CTAAGAATTTCACCTCTGACAGT-3'**.** Cycling conditions were similar to those used for *A. marginale* except for the annealing step which was performed at 60 °C for 1 min, and extension which was 72 °C for 30 s. The PCR products were subjected to electrophoresis on 2% agarose gels stained with ethidium bromide. The size of the amplified DNA fragments was estimated using a 100 bp molecular weight marker (Jena Bio-science GmbH) and the fragments were visualised under a UV transilluminator.

Medical management. Once tentative diagnosis was achieved, all of the diseased cattle were treated with Imidocarb (Imizol; Schering-Plough) delivered subcutaneously at a dose rate of 3 mg/kg body weight concomitantly with a single *i.m.* injection of long-acting oxytetracycline (Oxytetra, fatro Company, Italy) at a dosage of 20 mg/kg. For severely affected cases with PCV% below 15, blood transfusion was conducted to partially restore the PCV and to improve the survival rate. Ticks were controlled by spraying the diseased cattle as well as the surrounding environment with Deltamethrin (Butox, Intervet).

Statistical analysis. Data were statistically analysed for significant differences ($P < 0.05$) using a statistical software program (SPSS, version 16, USA). Differences between groups were compared using one-way ANOVA and Duncan's test. Means and standard deviations for each variable were esti-

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Table 1. Clinical variables in *A. marginale*-infected cattle (n = 39) compared to controls (n = 10)

Variables	Controls (n = 10)	Survivors (n = 26)	Non-survivors (n = 13)
Heart rate (beat/min)	57 ± 8.1[a]	96 ± 5.7[b]	120 ± 7.8[c]
Respiratory rate (cycle/min)	12.6 ± 2.0[a]	27 ± 4.1[b]	49.5 ± 9.4[c]
Rectal temperature (°C)	38.2 ± 0.34[a]	40.3 ± 0.5[b]	40.5 ± 0.4[b]
Cough	present (n = 0) absent (n = 10)	present (n = 19) absent (n = 7)	present (n = 11) absent (n = 2)
Dyspnoea	present (n = 0) absent (n = 10)	present (n = 11) absent (n = 15)	present (n = 11) absent (n = 2)
Mucous membrane colour	bright red (n = 10) congested (n = 0) pale (n = 0) icteric (n = 0)	bright red (n = 0) congested (n = 14) pale (n = 10) icteric (n = 2)	bright red (n = 0) congested (n = 0) pale (n = 7) icteric (n = 6)
Lymph nodes	enlarged (n = 0) normal (n = 10)	enlarged (n = 1) normal (n = 25)	enlarged (n = 1) normal (n = 12)
Oculonasal discharge	present (n = 0) absent (n = 10)	present (n = 23) absent (n = 3)	present (n = 11) absent (n = 2)
Haemoglobinurea	present (n = 0) absent (n = 10)	present (n = 0) absent (n = 26)	present (n = 3) absent (n = 10)
Bloody faeces	present (n = 0) absent (n = 10)	present (n = 6) absent (n = 20)	present (n = 10) absent (n = 3)
Appetite	normal (n = 10) inappetance (n = 0) anorexia (n = 0)	normal (n = 0) inappetance (n = 1) anorexia (n = 25)	normal (n = 0) inappetance (n = 0) anorexia (n = 13)
Posture	normal (n = 10) recumbency (n = 0)	normal (n = 26) recumbency (n = 0)	normal (n = 4) recumbency (n = 9)

[a,b,c] variables with different superscripts in the same column are significantly different at $P < 0.05$

mated. Chi square test was used to test the association between survival state of the animal and the observed clinical signs at P-value < 0.05. For biochemical parameters, data were tested for normality of distribution using the D'Agostino and Pearson Omnibus normality test. The Spearman correlation coefficient was also calculated to determine the correlation between parasitaemia rates and values of the selected acute phase cytokines and APPs.

RESULTS

Results of clinical findings and laboratory alterations in cattle with *A. marginale* infection compared to controls are illustrated in Tables 1–3 and Figures 1 and 2. *A. marginale* infection was initially diagnosed in 39 cattle on the basis of case history, clinical findings, and microscopic examination of blood smears, and then confirmed by PCR assays using species-specific primers (Figure 2). Using microscopy, *A. marginale* infections were identified in 30 of the diseased cattle. Clinically, the heart rates, respiratory rates, and rectal temperatures were significantly higher in non-surviving cattle

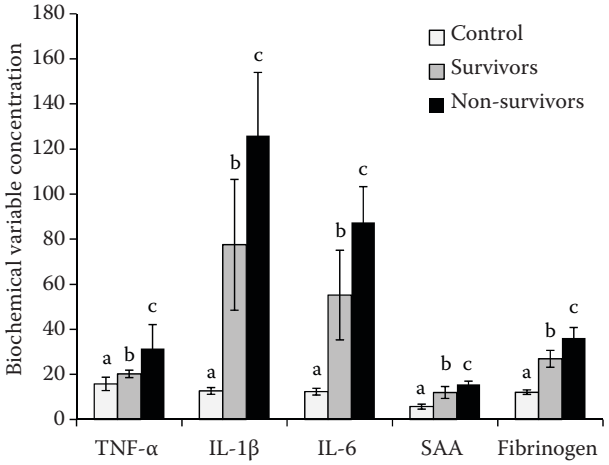

Figure 1. Mean values ± SD of selected pro-inflammatory cytokines and acute phase proteins in surviving and non-surviving cattle compared to controls. Bars labelled with different letters are statistically significant ($P < 0.05$)

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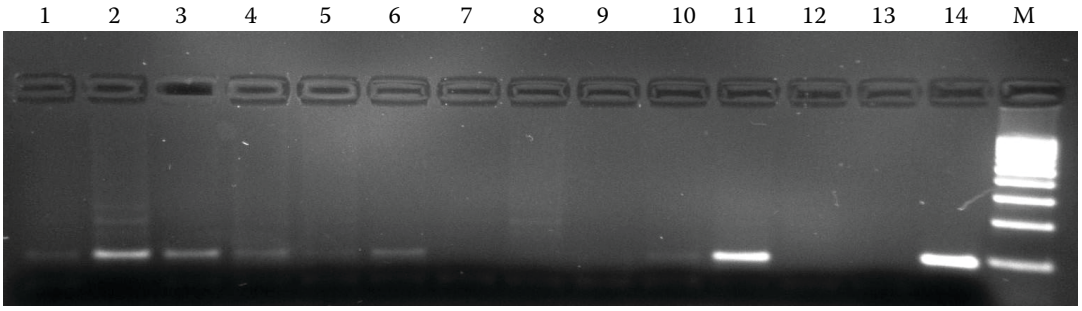

Figure 2. Agarose gel electrophoresis of the amplified PCR products using specific primers for *A. marginale.* Lanes 1–6, 10, 11 show 95 bp PCR products from field bovine blood samples; lanes 7, 8, 9, 12 represent negative samples for *A. marginale*; lane 13 is a negative control, lane 14 is positive control. Lane M is a DNA ladder (100 bp). The size of the marker (in bp) is shown on the right of the image

than survivors and controls ($P < 0.05$) (Table 1). There was also a significant association ($P < 0.05$) among non-surviving cattle and the following clinical variables with respective frequencies: cough ($\chi^2 = 20.32$, d.f = 2, $P < 0.0001$) (84.60%), dyspnoea ($\chi^2 = 16.51$, d.f = 2, $P < 0.0001$) (84.60%), haemoglobinurea ($\chi^2 = 8.85$, d.f = 2, $P = 0.012$) (23%), bloody faeces ($\chi^2 = 17.52$, d.f = 2, $P < 0.0001$) (76.9%), anorexia ($\chi^2 = 49.64$, d.f = 4, $P < 0.0001$) (100%), recumbency ($\chi^2 = 30.52$, d.f = 2 , $P < 0.0001$) (69.2%), and abnormal mucous membrane colour ($\chi^2 = 66.24$, d.f = 6, $P < 0.0001$) with high frequency in cattle with pale and icteric mucosa (53.9% and 46.1%), respectively. However, the surviving cattle were likely to develop congested mucous membranes ($\chi^2 = 66.24$, d.f = 6, $P < 0.0001$) and oculo-nasal discharge ($\chi^2 = 28.54$, d.f = 2, $P < 0.0001$) with high frequencies (53.8 and 88%, respectively).

Biochemically, TNF-α, IL1-β, IL-6, SAA, and fibrinogen were significantly higher in survivors and non-surviving cattle than in controls ($P < 0.05$), and were higher in non-survivors than survivors ($P < 0.05$) (Figure 1). The blood levels of an oxidative stress indicator (MDA) and anti-oxidant enzymes (including SOD and CAT) were significantly higher in the diseased cattle than in the controls ($P < 0.05$) and in non-survivors compared to survivors ($P < 0.05$). In contrast, G-SH was significantly lower in non-surviving cattle than in survivors and controls (Table 2). A significant correlation was found between parasitaemia of the diseased subjects and all of the measured biochemical variables. Among all of the measured variables, IL-1β and G-SH showed the highest correlation coefficient values ($r = 0.970$, $P < 0.01$) and ($r = -0.966$, $P < 0.01$), respectively. In addition, total

Table 2. Mean values ± SD of selected oxidative stress mediators and anti-oxidative variables in *A. marginale*-infected cattle ($n = 39$) compared to controls ($n = 10$)

Groups	MDA (nmol/ml)	SOD (IU/ml)	G-SH (mg/dl)	CAT (IU/l)
Control ($n = 10$)	5.33 ± 1.5^a	1996 ± 89^a	2.36 ± 0.37^a	267 ± 9.2^a
Survivors ($n = 26$)	13.05 ± 1.82^b	2192 ± 15^b	1.72 ± 0.3^b	534 ± 18.4^b
Non-survivors ($n = 13$)	18.25 ± 3.86^c	2805 ± 92^c	1.15 ± 0.06^c	658 ± 49^c

MDA = malondialdehyde, SOD = superoxide dismutase, G-SH = glutathione reduced, CAT = catalase
^{a,b,c} variables with different superscripts in the same column are significantly different at $P < 0.05$

Table 3. Mean values ± SD of parasitaemia, RBCs and PCV in *A. marginale*-infected cattle ($n = 39$) compared to controls ($n = 10$)

Groups	Parasitaemia (%)	RBCs (10^6/μl)	PCV (%)
Control ($n = 10$)	0.0^a	9.57 ± 0.5^a	38.6 ± 2.0^a
Survivors ($n = 26$)	7.25 ± 1.25^b	7.25 ± 0.5^b	26.25 ± 1.25^b
Non-survivors ($n = 13$)	16.0 ± 4.32^c	5.47 ± 0.9^c	18.5 ± 3.51^c

^{a,b,c} variables with different superscripts in the same column are significantly different at $P < 0.05$

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erythrocyte counts and PCV% were significantly lower in the diseased cattle than in controls and in non-survivors compared to survivors (Table 3).

DISCUSSION

In Egypt, bovine anaplasmosis represents a major constraint to livestock improvement programs, and causes serious health problems resulting in reduced animal productivity and economic losses. In the present study, *A. marginale* infection was initially diagnosed on the basis of clinical examination and confirmed with laboratory investigations. Animals suffering from acute anaplasmosis shared the following symptoms: fever, oculo-nasal discharge, frequent coughing, tachycardia, tachypnoea, and abnormal mucous membrane colour, decreased milk yield, decreased ruminal motility, and decreased faecal output. Although these symptoms are typical, they are not pathognomonic and can be confused with other tick-borne protozoan infections. Importantly, anorectic cattle and/or those with frequent coughing, dyspnoea, bloody faeces, and recumbency were most likely to die. The clinical examination alone was not enough to diagnose the underlying pathogen; therefore, it should be used together with other laboratory diagnostic methods. Nevertheless, we were able to identify *A. marginale* infection by microscopy in 76.92% of cases (18/26 survivors; 12/13 non-survivors) with a diagnostic accuracy of 97.2% and 99.6%, respectively. Nonetheless, the efficiency of traditional microscopic examination of Giemsa-stained blood films in detecting carrier animals with low rates of parasitaemia is very limited (Younis et al. 2009). The clinical findings presented in this study were in part similar to those previously described (De et al. 2012; OIE 2012; El-Ashker et al. 2015).

Over the past years, significant progress has been made in the application of APPs as biomarkers in several animal diseases; however, the prognostic significance of the different inflammatory and antioxidant markers in cattle naturally infected with *A. marginale* has not previously been addressed. In this study, we aimed to study inflammatory and oxidative stress biomarkers as a potential predictive tool for the outcome of anaplasmosis in cattle. Our results indicated that all of the measured variables were higher in cattle with *A. marginale* infection compared with controls, and were higher in non-survivors than survivors. The marked alterations in cytokines, APPs, and antioxidants in *A. marginale*-infected cattle suggest a high degree of induction of circulating inflammatory and oxidative stress responses during the course of infection. A similar APP response has been shown in a recent study (Nazifi et al. 2012). In previous reports it has been proposed that the lesions associated with anaplasmosis in cattle could drive the release of cytokines, which in turn induce the hepatic synthesis of APPs (Latimer et al. 2003).

It is believed that the pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 are required to initiate the inflammatory response to infection and tissue damage. It has also been reported that high concentrations of pro-inflammatory cytokines correlate with the prognosis of sepsis and the development of multiple organ dysfunctions (Edith et al. 2004). Moreover, the infusion of high concentrations of pro-inflammatory cytokines in rabbits was associated with multiple organ dysfunction syndrome (Kusawa et al. 1988). Several studies have suggested that the circulating levels of pro-inflammatory cytokines could be useful in assessing the severity and in predicting the clinical outcomes of some diseases (Rauchhaus et al. 2000; Orro et al. 2011; El-Ashker et al. 2013; El-Ashker et al. 2014).

The activities of antioxidant enzymes (SOD and CAT) and G-SH are considered to be good indicators for oxidative stress responses. Our data clearly demonstrated that *A. marginale*-infected cattle had higher levels of antioxidant enzymes and less G-SH compared to controls. This was in agreement with previous studies (Asri and Dalir 2006; Abdel Hamid et al. 2014). Moreover, we showed that the alterations in antioxidant levels were much more pronounced in non-survivors compared to survivors. It was previously reported that the antioxidant capacity of erythrocytes decreased with anaemia (Asri and Dalir 2006). The alteration in the activities of antioxidant enzymes could also be attributable to the accumulation of reactive oxygen species (ROS) leading to oxidative stress. The decreased G-SH observed in diseased animals could be due to either increased activity of glutathione peroxidase (GPX) that utilises G-SH as a source of reducing equivalents to reduce peroxides (da Silva et al. 2006) or due to its capacity to directly detoxify ROS (Freeman and Crapo 1982).

PCV%, erythrocyte count and haemoglobin levels were significantly decreased in infected cattle

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compared with controls. These results were similar to those reported by others (De et al. 2012; Abdel Hamid et al. 2014). It was suggested that the severe anaemia could be attributed to immune-mediated destruction of parasitised and non-parasitised erythrocytes (De et al. 2012).

In conclusion, our findings clearly demonstrate that A*naplasma* infection is associated with inflammatory and oxidative stress responses with high levels of the measured inflammatory and oxidative stress markers in non-surviving cattle compared with survivors. The extent of these responses could serve as an important prognostic indicator. Among the different biochemical variables examined, IL-1β and G-SH showed the highest correlation with parasitaemia levels and therefore, could serve as a biomarker for the inflammation and oxidative stress associated with *Anaplasma* infection. Moreover, the measured inflammatory and oxidative stress markers could constitute a useful tool in predicting the outcome of the disease.

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