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RESEARCH ARTICLE

Clinico-biochemical Investigation of Paratuberculosis of Dromedary Camels in Saudi Arabia: Proinflammatory Cytokines, Acute Phase Proteins and Oxidative Stress Biomarkers

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ABSTRACT

Searching for suitable biomarkers for diagnosis of *Mycobacterium avium subspecies paratuberculosis* is the key for control of Johne's disease. In the current study, two sets of twenty camels each, one infected with paratuberculosis and other normal healthy were executed. The basis for the infection was positive findings of clinical examination, Ziehl Neelsen staining of rectal smear and PCR of extracted bacterial DNA from feces. The present findings revealed significant decrease in total erythrocyte counts (TEC) and hemoglobin concentration (Hb) with significant increase in total leucocytes counts (TLC), packed cell volume (PCV) and neutrophils percentages of infected camels compare to control. A significant decrease in values of total proteins, albumin and glucose with significant elevation of concentration of blood urea nitrogen (BUN) and bilirubin and enzyme activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyle transferase (GGT) and Glutamic acid dehydrogenase (GLDH) were noted in the serum of infected camels compare to control. Acute phase proteins (APP) namely, haptoglobin (Hp), serum amyloid A (SAA) and fibrinogen (Fb) and proinflammatory cytokines namely, interleukins (1α, 1β, 10, 6), tumor necrosis factor α (TNF-α) and interferon gamma (INF-γ) were significantly increased in infected camel compare to control. Significant decrease in the levels of oxidative stress biomarkers namely, superoxide dismutase (SOD), glutathione concentration (GSH) and catalase (CAT) with significant increase in the levels of Malondialdhyde (MDA) were observed in infected camel compare to control. The present study suggests APP, proinflammatory cytokines and oxidative stress parameters as additional biomarkers for paratuberculosis in camel.

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INTRODUCTION

The camel is the most efficient domesticated animal for converting fodder into work, transport, milk and meat (Hamad *et al*., 2011; Samara *et al*., 2012; Pasha *et al*., 2013). Although, several reports demonstrated that camels is less susceptible to disease than other livestock (Saint-Martin *et al*., 1992; Farooq *et al*., 2012), camels are more susceptible than other animals to paratuberculosis (Wernery and Kaaden, 2002). Paratuberculosis is a chronic debilitating granulomatous enteritis of domestic and wild ruminants mainly, cattle, sheep, goats, camel, deer, antelope and bison. The disease is caused by *Mycobacterium avium subspecies paratuberculosis* (Collins, 2003). Clinically, the disease in ruminants is characterized by irreversible wasting, diarrhea, dehydration and later may be death. The infection is chronic, progressive and unresponsive to treatment (Yayo *et al*., 2001). In Saudi Arabia, paratuberculosis was documented in different ruminant specie namely cattle,

sheep, goat and camel (Alharbi *et al*, 2012; Almujali and Al Ghamdi, 2012). Although, the clinical picture of the disease was well documented in domesticated ruminants rather than camel, in the last few years, it was observed that paratuberculosis is a problem in many camel herds in the Kingdom. The general circumstantial evidences are increased clinical cases, samples from abattoirs, owner's observations and the veterinarians examination (Almujali and Al Ghamdi, 2012; Al Ghamdi, 2013). Few reports (Almujali and Al Ghamdi, 2012; Salem *et al*., 2012; Al Ghamdi, 2013) investigated the effect of paratuberculosis on hematological and biochemical parameters of dromedary camels. The authors (Almujali and Al Ghamdi, 2012; Al Ghamdi, 2013) demonstrated that the results revealed a significant increase in creatinine, blood urea nitrogen, magnesium, AST and ALT in diseased camels. The same authors reported that, glucose, total protein and albumin were significantly decreased in diseased camels when compared to healthy ones. In addition, thickening corrugation of the intestinal wall, enlarged folded mucosa and oedemated ileocaecal and mesenteric lymph nodes were also reported. Successfully, proinflammatory cytokines and oxidative stress parameters were introduced by scientific community as useful biomarkers in the diagnosis of rhabdomyolysis in equine (El-Deeb and El-Bahr, 2010) and bronchopneumonia in water buffalo (El-Bahr and El-Deeb, 2013). However, publications regarding the effect of paratuberculosis on proinflammatory cytokines, acute phase proteins and oxidative stress markers in camels are lacking. Therefore, the present study aimed to investigate this as a trial to present additional biomarkers for John's disease in camels.

MATERIALS AND METHODS

Animals and clinical examination: Twenty camels (2-4 years old) presented to Veterinary Hospital, King Faisal University, Saudi Arabia were found infected with paratuberclosis based on positive findings of clinical examination, Ziehl Neelsen staining of rectal smear and PCR of extracted bacterial DNA from feces. Another 20 normal camels from Camel Research Centre, King Faisal University were used as control. The diseased animals showed watery diarrhea for more than two months. Moreover, signs of weakness, emaciation, fluctuating temperature and dehydration were also reported.

Collection of samples: Fecal samples were collected from diseased animals and kept frozen at -80°C until the time of PCR analysis. Blood samples were collected from the jugular vein of both groups into plain and EDTA vacutainers.

DNA extraction and PCR amplification: Total DNA was isolated from fecal samples by using the QIAamp DNA stool Mini Kit (QIAGEN) according to the manufacturer`s recommendation.

Paratuberculosis extracted DNA was amplified by PCR using primers $P90^{+}$(5′- GAAGGGTGTTCGGGGCC GTCGCTTAGG-3′) and $P91^{+}$(5′- GGCGTTGAGGTCGA TCGCCCACGTGAC-3′) (Millar *et al*., 1996). PCR amplification was performed in a total volume 20µl of HotStartTaq® Plus Master Mix Kit (2x) "QIAGEN". The PCR cycling profile consisted of one cycle for initial heat activation of 95°C for 5 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minutes and final cycle for 10 minutes at 72°C. 10 µl of each amplified product were analyzed by agarose gel electrophoresis on 1.5% agarose containing 0.5µg /ml ethidium bromide and visualized by gel documentation system.

Hematological and biochemical analysis: Complete blood picture was determined using electronic cell counter (VetScan HM5 Hematology system). The concentration of total protein, albumin, glucose, BUN, Bilirubin and activities of ALT, AST, ALP, GGT and GLDH were measured in serum samples on a Beckman CX-7 autoanalyser using commercial kits (Sigma Chemical Co. Ltd., Poole, Dorset, UK). Fb concentration in plasma was measured with a commercially available ELISA kit (USCA, Life Science) according to the manufacturer's instructions. Serum Hp was determined using the hemoglobin binding assay described by Makimura and Suzuki (1982). SAA was measured with a commercially available ELISA kit (Phase SAA kit, Tridelta Ltd., Ireland), according to the manufacturer's instructions. Levels of MDA, R.GSH, CAT and SOD activities were determined using commercial kits according to the methods described by Ohkawa *et al*. (1979), Beutler *et al*. (1963), Cowell *et al*. (1994) and Nishikimi *et al*. (1972), respectively. IL-1α, IL-1β, IL-10, IL-6, TNF-α, and IFN-γ levels were determined from undiluted serum samples using commercially available ELISA Kits (BioSource, Diagnostic Corporation, Belgium).

Statistical analysis: All data was presented as mean±SE of mean by using student-t-test. All tests were performed using computer package of the statistical analysis system (SAS, 2002).

RESULTS

Clinical signs: The diseased camels showed persistent diarrhea, anorexia and weight loss. Signs of weakness, emaciation, fluctuating temperature and dehydration were also noted.

PCR amplification: The total DNA extracted from fecal samples was strongly positive with the expected size corresponding to mycobacterium paratuberculosis by IS900 PCR (Fig. 1).

Hematological analysis: Data summarized in Table 1 indicated a significant ($P \leq 0.05$) decrease in the levels of TEC and Hb with significant increase ($P \leq 0.05$) in TLC, PCV and neutrophils percentage of infected camels compare to control. The percentages of lymphocytes, monocytes, basophils and eosinophil were unchanged significantly ($P > 0.05$) in infected camel compare to the control.

Biochemical analysis: The present findings (Table 2) indicated a significant decrease ($P \leq 0.05$) in total proteins, albumin and glucose in the serum of infected camels

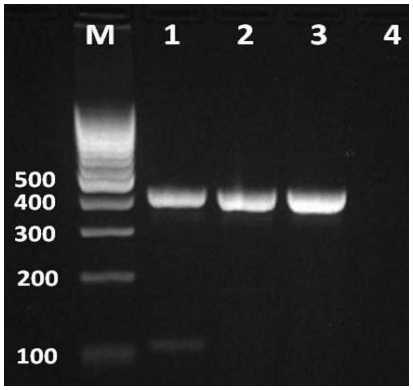

Fig. 1: Ethidium bromide-stained agarose gel showing the approximately 400bp *Mycobacterium paratuberculosis* band of PCR products (expected site). Lane M: 100bp molecular weight markers; Lanes 1 and 2: fecal samples of two suspected paratuberculosis infected cases 3; DNA positive control of *Mycobacterium paratuberculosis* and Lane 4: Negative control DNA.

compare to the control. Significant elevation (P≤0.05) in BUN and Bilirubin concentrations was also noted in infected camels compare to control. All estimated enzyme activities of AST, ALT, ALP, GGT and GLDH were significantly elevated (P≤0.05) in the serum of infected camels compare to control (Table 2).

Oxidative stress biomarkers: The results shown in Table 3 indicated that, the activities of SOD and CAT and RGSH level were reduced significantly (P≤0.05) in infected camels compare to the control. Lipid peroxidation was increased significantly (P≤0.05) as reflected on higher MDA value in the serum of infected camels compare to control.

Acute phase proteins and proinflammatory cytokines: The data summarized in Table 4 showed that APP, Hp, SAA and Fb were significantly (P≤0.05) increased in the serum of infected camel compared to control. In addition, the values of all pro-inflammatory cytokines, IL-1α, IL-1β, IL-6, IL-10, TNF-α and IFN-γ were also increased significantly (P≤0.05) in paratuberculosis infected camels compare to control.

DISCUSSION

No doubt, the effective diagnosis is important for successful disease control program to avoid expensive treatment. Hence, additional biochemical markers are essential for control of paratuberculosis in dromedary camels. Most recently, haematological and biochemical indices were used as diagnostic tools of Johne's disease in dromedary camels (Salem *et al*., 2012; Almujali and Al Ghamdi, 2012; Al Ghamdi, 2013). In the current study, normal values for hematological parameters were in concurrent of reference ranges for the hematological parameters of dromedary camels (AL-Busadah, 2007; Salem *et al*., 2012; Almujali and Al Ghamdi, 2012; Al Ghamdi, 2013). The deviation of hematological parameters from their reference value often provides valuable information regarding the health status of the animals which could be taken in consideration during diagnosis and treatment of camel diseases (Kamal, 2008).

Table 1: Mean hematological values of healthy and paratuberculosis infected dromedary camels

Parameters	Units	Healthy camels (Control)	Diseased camels
Total erythrocyte count	10^6/ul^3	11.15±3.22	8.45±0.22*
Hemoglobin	g/dl	8.98±0.08	7.78±0.12*
Packed cell volume	%	46.3±0.08	48.4±0.06*
Total leucocyte count	X 10^3/ul^3	14.20±5.31	24.20±4.11*
Neutrophils	%	57.0±3.44	82.0±3.56*
Lymphocytes	%	25.3±3.56	24.3±1.26
Monocytes	%	2.4±0.32	2.3±0.22
Basophils	%	1.1±0.12	1.0±0.13
Eosinophils	%	2.4±0.21	2.6±0.32

Mean±SE values bearing asterisk in a row differ significantly (P≤0.05).

Table 2: The mean values of blood biochemical parameters of healthy and paratuberculosis infected dromedary camels

Variables	Units	Healthy camels	Diseased camels
Total Proteins	g/dl	6.34±0.14	5.10±0.15*
Albumen	g/dl	3.74±0.2	3.00±0.2*
Blood urea nitrogen	g/dl	12.50 ±0.34	21.20±3.4*
Glucose	mmol/l	37.67±3.10	30.50±2.5*
Bilrubin	mmol/l	1.10±0.05	1.90±0.05*
Aspartate transaminase	mmol/l	139.50±23.4	197.21±21.4*
Alanine transaminase	mmol/l	17.30±4.9	55.11±5.5*
Alkaline phosphatase	IU/l	45.60±2.6	88.20±4.5*
Gamma glutamyle transferase	IU/l	15.63±2.2	66.30±6.35*
Glutamate dehydrogenase	IU/l	13.56±5.21	55.45±6.8*

Mean±SE values bearing asterisk in a row differ significantly (P≤0.05).

Table 3: The mean values of oxidative stress biomarkers of healthy and paratuberculosis infected dromedary camels

Parameters	Units	Healthy camels (Control)	Diseased camels
Malondialdhyde	nmol/g Hb	12.5±1.21	26.6±1.23*
Superoxide dismutase	U/mg Hb	5.14±0.45	3.52±0.22*
Catalase	U/mg Hb	16.77±1.23	6.32±0.85*
Reduced glutathione	mmol/gm Hb	7.5±0.56	3.8±0.48*

Mean±SE values bearing asterisk in a row differ significantly (P≤0.05).

Table 4: The mean values of acute phase proteins and proinflammatory cytokines of healthy and paratuberculosis infected dromedary camels

Parameters	Units	Healthy camels (Control)	Diseased camels
Haptoglobin	g/l	0.3±0.03	1.2±0.04*
Serum amyloid A	mg/l	9.5±0.40	27.6±2.20*
Fibrinogen	g/l	3.3±0.30	5.0±0.20*
Tumor necrosis factor-α	pg/ml	14.45±0.54	23.25±0.43*
Interferon-γ	pg/ml	18.65±1.23	34.23±1.25*
Interleukin-1α	pg/ml	9.23±0.23	15.21±0.23*
Interleukin-1β	pg/ml	19.32±1.84	27.23±0.52*
Interleukin-6	pg/ml	13.54±0.89	17.23±0.32*
Interleukin-10	pg/ml	9.92±0.32	15.54±0.23*

Mean±SE values bearing asterisk in a row differ significantly (P≤0.05).

The significant decrease of TEC and Hb came in accordance of previous reports (Salem *et al*., 2012; Almujali and Al Ghamdi, 2012; Al Ghamdi, 2013) demonstrated the same effect in dromedary camels. These findings indicated the presence of anemia among the infected camels (Weiss and Goodnough, 2005). The significant increase in TLC and neutrophil observed in the present study were combatable with previous reports (Chaudhary and Iqbal, 2000; Salem *et al*., 2012; Almujali and Al Ghamdi, 2012; Al Ghamdi, 2013) demonstrated the same effect in mice and racing camels, respectively.

The significant decrease in total proteins and albumin (Table 2) agrees with that previously published articles (Kamal, 2008; Salem *et al*., 2012; Almujali and Al Ghamdi, 2012; Al Ghamdi, 2013) in dromedary camels. The hypoproteinemia might be attributed to decrease of

albumin. The significant decrease of albumin might be due to leakage of albumin through damaged tissues and the increase of gamma globulins is a compensatory reaction to restore the reduced plasma osmotic pressure resulted from loss albumin. This can explain the obtained significant decrease in serum albumin of infected camels. The significant increase in AST, ALT, ALP, GGT and GLDH activities in serum of infected camels than normal camels indicated liver affection and give interpretation to decreased total proteins and albumin values in infected camels compare to healthy animals. Similar results were obtained in dromedary camels suffered from gastrointestinal inflammatory disorders (Bengoumi *et al.,* 1997; Kamal, 2008). As a result of liver affection, bilirubin concentration was increased and glucose level was decreased significantly in infected camel than normal camels. The hypoglycemia noted in the present study agrees with previous reports explained that, animals suffering from gastrointestinal infection usually exhibits hypoglycemia (Weiss and Goodnough, 2005). The significant increase in BUN agree with the results obtained earlier in dromedary camels suffered from Johne's disease (Almujali and Al Ghamdi, 2012; Al Ghamdi, 2013).

In the current study, the significant increase of MDA in the serum of infected camels indicated that lipid peroxidation has been exhibited as results of leakage of reactive oxygen species (ROS) (Yousef *et al*., 2009). These ROS might be produced as a results of bacterial infection (paratuberculosis) and if are not removed by endogenous enzymatic (SOD, CAT) and non-enzymatic (RGSH) antioxidant defenses of the organism, the oxidative stress will be produced as showed in the present findings (Sies, 1991).

The significant increase of proinflammatory cytokines in the serum of infected camels in the present study demonstrated the stimulation of monocytes and macrophages to produce proinflammatory cytokines (TNF-α, IL1β, IL-10 and IFN-γ), to withstand the bacterial infection. The significantly elevated proinflammatory cytokines mediated the effect of positive APP (SAA, Fb and Hp) which increased significantly also in infected camels to favor T-helper cell differentiation that construct abridge between innate resistance and adaptive immunity (Trinchieri, 2003). The present findings were parallel to previous works demonstrated that paratubeculosis infection was controlled with high levels of IFN-γ (Chaudhary and Iqbal, 2000). Parallel to the present study, earlier report (Berg *et al*., 1996) demonstrated that, IL-10 deficient mice develop spontaneous murine colitis and weight loss with marked increase in APP. Additionally, other report (Singh *et al*., 2007) stated that, the components of paratuberculosis might enhance the production of IFN-γ and TNF-α in human.

Conclusion: The present data provide the first evidence of elevated oxidative stress biomarkers, acute phase proteins and proinflammatory cytokines in dromedary camels infected with *Mycobacterium avium subspecies paratuberculosis*.

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