Cardiac and oxidative stress biomarkers in *Trypanosoma evansi* infected camels: diagnostic and prognostic prominence

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(*Received 23 October 2014; revised 20 November 2014; accepted 21 November 2014; first published online 12 January 2015*)

SUMMARY

This study was conducted to investigate the level of cardiac and oxidative stress markers in camels infected with *Trypanosoma evansi* and to explore the diagnostic and prognostic value of cardiac troponin I (cTnI) and creatine kinase-myocardial band (CK-MB) in response to infection. Seventy four dromedary camels with clinical and laboratory evidence of trypanosomosis and 20 healthy controls were included in this study. Serum cTnI, CK-MB, CK, malondialdehyde (MDA) and super oxide dismutase (SOD) were measured. The values of cTnI, CK-MB, CK and MDA were significantly higher, whereas SOD level was lower in *T. evansi* infected camel. Successfully treated camels ($n = 43$) had lower levels of cTnI, CK-MB, CK and MDA, but higher level of SOD compared to camels with treatment failure. Both cTnI and CK-MB showed high degree of accuracy in predicting treatment outcome (success *vs* failure). The area under the curve for cTnI and CK-MB was 0·98 and 0·93, respectively. However, cTnI showed better sensitivity and specificity than CK-MB (Se = 96·8% *vs* 83·9% and Sp = 100% *vs* 88·5%, respectively). These results suggest that cTnI and CK-MB could be used as diagnostic and prognostic biomarkers in camels infected with *T. evansi*.

Key words: camels, *Trypanosoma evansi*, cardiac troponin I, creatine kinase-MB, oxidative stress.

INTRODUCTION

Trypanosoma evansi (T. evansi) is the causative agent of trypanosomosis, a widely spread disease, affecting many wild and domestic animals species including camels (Luckins and Dwinger, 2004). The disease causes high economic losses in camel industry due to high morbidity, mortality, low milk and meat production, poor carcass quality and reduced reproductive performance (Pacholek *et al.* 2000; Njiru *et al.* 2002). The disease occurs in acute and chronic forms, however, chronic form is more prevalent and manifested by anaemia, emaciation, lacrimation, lymphadenitis and sometimes abortions (Gutierrez *et al.* 2005). Anaemia resulting from trypanosomosis is primarily haemolytic and has been attributed to either dyshematopoiesis or erythrophagocytosis (Anosa, 1988). Anaemia followed by secondary hypoxia has been suggested to induce cardiac damage (Fartashvand *et al.* 2013).

Cardiac troponin I (cTnI), is a regulatory protein controlling calcium-mediated interaction between actin and myosin and is considered a highly sensitive and precise biomarker for diagnosis of myocardial damage in humans (Reagan *et al.* 2013) and animals (Wells and Sleeper, 2008; Fonfara *et al.* 2010). The values of serum cTnI increase in response to cardiac injury as a consequence of enzyme leakage from cardiac cells (O'Brien *et al.* 2006). Several studies reported an increase in the level of cTnI in response to leishmaniosis (Silvestrini *et al.* 2012), and babesiosis (Lobetti *et al.* 2012) in dogs, bovine theileriosis (Fartashvand *et al.* 2013) and in camels with tick infection (Tharwat and Al-Sobayil, 2014). Moreover, the level of cTnI has been also used as a marker of cardiac injury during camel transportation (Tharwat *et al.* 2013). Another biomarker of cardiac injury is creatine kinase (CK) enzyme which exists as isoenzymes in three combinations: MM, MB and BB. The CK isoenzymes is distributed in many tissues including skeletal muscles, but there is more of CK-MB fraction in the heart, therefore CK-MB is considered relatively specific for myocardial injury when skeletal muscle damage is absent (McLean and Huang, 2012).

In dromedary camels, many cardiac problems cannot be diagnosed except after necropsy, therefore there is a need for introducing non-invasive diagnostic methods for such problems.

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Parasitology (2015), **142**, 767–772.
doi:10.1017/S0031182014001899

Recently, camel trypanosomosis has been linked with a state of oxidative stress process, with significant increase of serum malondialdehyde (MDA), reduction in super oxide dismutase (SOD) and no change in catalase (Saleh *et al*. 2009). The values of MDA in blood and tissues are commonly used as biomarkers of lipid peroxidation (Yousef *et al*. 2009). The SOD is an antioxidant enzyme which protects the tissue against oxidative stress by reducing superoxide anion to hydrogen peroxide, which is then converted to water by catalase and glutathione peroxidase (Rahman *et al*. 2006). Oxidative stress has been also reported to play an important role in both myocardial injury and repair (Ansley and Wang, 2013).

The objectives of the present study were: to evaluate the serum concentration of cTnI, CK-MB in camels infected with *T. evansi* infection as a marker of possible myocardial injury, to investigate diagnostic and prognostic accuracy of cTnI and CK-MB in infected camels, and to monitor the relationship between cTnI, blood picture, oxidative stress markers, CK-MB and CK in infected camels.

MATERIALS AND METHODS

Study animals

A total of 94 camels (52 male [mean age = 6·1 year] and 42 female [mean age = 5·6 year]) admitted to Veterinary Teaching Hospital, King Faisal University, Saudi Arabia during the period from September 2012 to December 2013 were included in the present study. Camels were divided into two groups based on clinical, haematological and parasitological examination. The first group consisted of 20 clinically healthy camels (12 male and 8 female) that were admitted to the Teaching Hospital for a routine examination before breeding season. Based on the laboratory results, the selected camels were tested negative for presence of other parasites. The second group consisted of 74 camels (40 male and 34 female) with clinical picture of trypanosomosis. All camels were thoroughly examined and clinical signs were carefully recorded.

The infection in camels was confirmed by the presence of the parasite in blood using wet-blood film and also micro-Haematocrit technique (OIE Manual, 2012). Positive camels were also examined by stained blood smears (Diff-Quick®). Moreover, latex agglutination test (LAT) was applied on all examined camels according to the method described by (Olaho-Mukani *et al*. 1996). The infected camels were consequently treated with trypanocidal preparations (Cymelarsan®, Merial). Forty three of 74 camels with trypanosomosis were successfully treated. Blood samples were collected from the jugular vein of both groups into plain and heparin vacutainers. Whole blood was used for the determination of haemoglobin content (Hb), total erythrocytic count (TEC), packed cell volume (PCV), red blood cell indices, total and differential leucocytic counts. Sera were harvested and stored at −20 °C until assayed for cTnI, CK-MB, CK, MDA and SOD. Fecal samples were also collected from all selected camels and were analysed using standard sedimentation-flotation technique according to the methods described by Coles (1980).

Haematological and biochemical analysis

Complete blood picture was determined using electronic cell counter (VetScan HM5 Hematology system).Cardiac troponin I was analysed in serum samples using a point-of-care analyser (VetScan i-STAT® 1, Abaxis, CA, USA) according to the manufacturer's instructions. All results are expressed as nanograms per milliliter (ng mL^{-1}) with an intra- and inter-assay coefficient of variance of 5·0 and 5·7%, respectively. The analysis of CK-MB was carried out using the Roche Diagnostics Cobas 6000 c501, electrochemiluminescent assay (Roche, Indianapolis, Indiana, USA). CK was analysed using VetScan VS2 chemistery analyser (Abaxis, California, USA).The SOD activity was assessed in the RBC haemolysate according to the method described by Misra and Fridovich (1972).

For MDA, lipid peroxidation in RBC haemolysate and serum was estimated as thiobarbituric acid reactive substances (TBARS) according to Placer *et al*. (1966). Lipid peroxidation in the RBC haemolysate was expressed as nmol of erythrocytic malondialdehyde (eMDA) per g Hb. Lipid peroxidation in serum was expressed as *nmol* of serum malondialdehyde (sMDA) per g serum protein (Shimadzu AA-6800 atomic absorption spectrophotometer, Koyoto, Japan).

Statistical analysis

Because of the small size of the control group and non-normally distributed markers in camels with *T. evansi*, differences in blood biomarkers between camels with treatment success or failure were compared using non-parametric analysis (Wilcoxon Mann–Whitney) at *P value* <0·05. Selection of cut-off points that optimize sensitivity (Se) and specificity (Sp) for each of cTnI and CK-MB was determined using receiver operating characteristics (ROC) analyses. The ROC curves were constructed by plotting sensitivity *vs* 1-specificity (false-positive rate) for all possible cut-off points for cTnI and CK-MB. The area under the curve (AUC) indicates the overall accuracy of the tested parameter. The difference in the AUC for the cTnI and CK-MB was compared using non parametric method which account for correlation resulting from using the same samples for both tests (DeLong *et al*. 1988). All

https://doi.org/10.1017/S0031182014001899 Published online by Cambridge University Press

analyses were done using Stata version 13 (Stata Corp, College Station TX, USA).

Ethics statement

All the animal procedures were performed according to the guidelines of the Animal Ethics Committee of College of Veterinary Medicine and Animal Resources, King Faisal University, Saudi Arabia.

RESULTS

All of the diseased camels showed progressive anaemia, pale mucous membrane, emaciation, marked depression or lethargy 'upward hanged head, called locally Heyam', recurrent fever, dullness, enlargement of lymph nodes and lacrimation. Atrophy of the thigh muscles was observed in (42/74), corneal opacity (21/74), diarrhoea (28/74), oedema of the dependent parts (48/74), loss of condition and some cases showed nervous signs (22/74) such as trembling, and unusual aggressiveness. Pointed shape feces and bitter odour of urine were also typical for the infected camels.

Data summarized in Table 1 indicates a significant ($P < 0{\cdot}05$) reduction in the values of TEC, Hb content and PCV% with significant elevation ($P < 0{\cdot}05$) in mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total leucocytic count (TLC), neutrophils, lymphocytes, monocytes and eosinophils counts in infected camels compared to control.

Table 2 shows variations in haematological parameters between successfully treated camels and those with treatment failure. The response to treatment was significantly ($P < 0{\cdot}01$) associated with RBCs count, Hb content, PCV%, mean corpuscular volume (MCV), MCH, MCHC and TLC count.

Table 3 shows that infected camels had significantly ($P < 0{\cdot}0001$) higher values of cTnI, CK-MB, CK, sMDA and eMDA than healthy controls. In contrast, a lower level of SOD was observed in infected camels compared to control ones. Data presented in Table 4 describes the variables depending on treatment success or failure. The response to treatment was significantly ($P < 0{\cdot}05$) associated with cTnI, CK-MB, CK, sMDA, eMDA and SOD levels.

Spearman's correlation (r) analysis showed high negative ($r \geq -0{\cdot}82$, $P < 0{\cdot}001$) correlation between treatment response and each of cTnI and CK, whereas RBC, Hb and TLC were highly and positively ($r \geq 0{\cdot}85$, $P < 0{\cdot}001$) correlated with treatment response. Additionally, cTnI was positively correlated with each of CK-MB ($r = 0{\cdot}70$), serum CK ($r = 0{\cdot}81$) and sMDA ($r = 0{\cdot}30$). On the other hand, the values of cTnI were inversely correlated with the values of RBC ($r = -0{\cdot}75$), Hb ($r = -0{\cdot}72$), TLC ($r = -0{\cdot}44$) and SOD ($r = -0{\cdot}58$).

From Table 5 and Fig. 1, both cTnI and CK-MB showed high accuracy in predicting treatment response, at the selected threshold (AUC = 0·98 and 0·93, respectively). However, cTnI showed better sensitivity (Se = 96·8% *vs* 83·9%) and specificity (Sp = 100% *vs* 88·5%) than CK-MB. Comparison of the AUC indicated no significant difference between AUC for cTnI and AUC for CK-MB ($P = 0{\cdot}153$).

DISCUSSION

To the best of authors' knowledge, this is the first study to address the diagnostic and prognostic accuracy of cTnI and CK-MB in cases of *T. evansi* infection in camels. The mean serum cTnI of the control camels in this study was similar to that recently reported in healthy camels (Tharwat *et al*. 2013). In the current investigation, higher values of cTnI in camels with *T. evansi* infection may indicate a possible myocardial injury. In the successfully treated camels, the cTnI was significantly lower than camels with treatment failure.

The cTnI is considered the 'gold-standard' biomarker with a great sensitivity and specificity in diagnosis of cardiac disorders (O'Brien *et al*. 2006; Wells and Sleeper, 2008). Such specificity of cTnI could be related to its tissue specificity, low basal blood concentration, prompt release and detection in the blood. The serum levels of cTn has been shown to be correlated well with histopathological changes in the myocardium, cardiac pathophysiology; degree of heart damage; clinical picture and disease outcome (O'Brien *et al*. 2006; Wells and Sleeper, 2008; Fonfara *et al*. 2010). The precise mechanism of cardiac injury in camels with *T. evansi* infection is uncertain. However, elevated serum cTnI concentration in camels with *T. evansi* infection may be attributed to the occurrence of anaemia, which was evident in the current investigation, as indicated by the lowered RBC count, haemoglobin concentration and haematocrit percent as well as the high negative correlation between cTnI and each of RBC count and Hb content. The obtained results support previous hypothesis that anaemia, followed by hypoxia may lead to cardiac damage (Fartashvand *et al*. 2013). The same authors also found that anaemic cattle with theileriosis had higher cTnI level than healthy cattle.

In the present study, cTnI concentration was $<1{\cdot}23$ ng mL^{-1} in successfully treated camels. On the other hand, 30 out of 31 camels with treatment failure had a serum cTnI concentration $>3{\cdot}54$ ng mL^{-1}. Therefore, it can be suggested that the increased serum concentration of cTnI over 3.54 ng mL^{-1} at initial examination was a bad prognostic indicator in camels with *T. evansi* infection. The cTnI showed a great Se and Sp in predicting treatment failure (Se 96·8%, Sp 100% and AUC = 0·98).

https://doi.org/10.1017/S0031182014001899 Published online by Cambridge University Press

Table 1. Descriptive results of haematological parameters in control and *T. evansi* infected camels and their statistical significance

	Control camels ($N = 20$)				Infected camels ($N = 74$)				
Parameter	Mean	Median	Min	Max	Mean	Median	Min	Max	*P*-value*
RBCs (10^3 mm^{-3})	11·54	11·55	10·50	12·50	7·00	7·45	4·50	9·40	0·0001
Hb (gm dL^{-1})	13·92	14·23	13·26	14·40	7·73	7·62	5·25	13·87	0·0001
PCV (%)	28·53	28·45	27·60	29·50	8·61	9·40	3·80	11·50	0·0001
MCV (fl)	24·70	24·71	23·40	25·70	28·38	24·70	21·45	43·20	0·8715
MCH (pg)	14·19	14·21	12·30	16·44	22·58	19·48	16·45	35·45	0·0001
MCHC (g dL^{-1})	55·43	56·14	48·12	61·45	62·44	60·15	50·14	74·54	0·0001
WBCs (×10^9 L^{-1})	15·27	15·44	14·15	16·45	16·81	17·85	10·25	20·15	0·0001
Neutrophils (×10^9 L^{-1})	10·66	10·60	9·50	11·50	16·81	17·50	10·25	19·60	0·0001
Lymphocytes (×10^9 L^{-1})	5·81	5·55	4·50	7·50	3·44	3·50	2·30	8·50	0·0001
Monocytes (×10^9 L^{-1})	0·02	0·02	0·01	0·04	0·03	0·03	0·01	0·05	0·0051
Eosinophils (×10^9 L^{-1})	0·24	0·24	0·23	0·24	0·74	0·78	0·23	0·99	0·0001

MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.
* *P*-value resulting from non-parametric Wilcoxon Mann–Whitney test.

Table 2. Descriptive statistics of haematological parameters in *T. evansi* infected camels with treatment success and failure

	Treatment success ($N = 43$)				Treatment failure ($N = 31$)				
Parameter	Mean	Median	Min	Max	Mean	Median	Min	Max	*P*-value*
RBCs (10^3 mm^{-3})	8·09	8·20	6·90	9·40	5·49	5·50	4·50	6·50	0·0001
Hb (gm dL^{-1})	8·05	8·20	5·42	13·87	7·28	7·36	5·25	13·78	0·0089
PCV (%)	10·19	10·60	5·50	11·50	6·47	6·50	3·80	8·50	0·0001
MCV (fl)	23·45	23·45	21·45	25·63	35·21	35·15	24·15	43·20	0·0001
MCH (pg)	18·34	18·45	16·45	20·14	28·47	29·45	18·90	35·45	0·0001
MCHC (g dL^{-1})	60·16	60·14	57·15	66·12	65·72	67·47	50·14	74·54	0·0001
WBCs (×10^9 L^{-1})	18·69	18·45	16·12	20·15	15·58	15·45	14·15	16·45	0·0001
Neutrophils (×10^9 L^{-1})	16·73	17·36	10·25	19·50	16·93	17·50	10·50	19·60	0·5421
Lymphocytes (×10^9 L^{-1})	3·46	2·80	2·30	2·80	3·42	3·50	2·40	3·50	0·1944
Monocytes (×10^9 L^{-1})	0·03	0·03	0·02	0·04	0·03	0·030	0·01	0·05	0·0841
Eosinophils (×10^9 L^{-1})	0·76	0·78	0·23	0·98	0·72	0·74	0·23	0·99	0·286

MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.
* *P*-value resulting from non-parametric Wilcoxon Mann–Whitney test.

Wang *et al*. (2009) conducted a study on the prognostic accuracy of cTnI in patients with non-primary cardiac disorders and concluded a positive predictive ability for cTnI on the disease outcome. A significant elevation in the values of CK-MB was also observed in *T. evansi* infected camels. The elevated levels of CK-MB could be attributed to skeletal muscle injury caused by *T. evansi* and possibly myocardial injury. Furthermore, the CK-MB showed a relatively good Se and Sp in predicting treatment outcome (Se 83·9%, Sp 88·5%). The higher sensitivity and specificity of cTnI compared to CK-MB may be due to the presence of high concentration of troponin in heart cells. In addition, most of depleted cTnI resulting from cardiac injury released in the blood. On the other side, CK-MB is not specific for cardiac injury as a small amount is found in skeletal muscles.

Other possible mechanism of cardiac injury is the state of oxidative stress in infected camels with *T. evansi*. Oxidative stress increases in cases with chronic heart failure and have been linked with myocardial disorders. An obvious mechanism through which myocardial oxidative stress might hinder cardiac activities is through oxidative injury to cellular proteins and membranes (Ansley and Wang, 2013).

In the present study, oxidative stress was evident as indicated by higher level of lipid peroxidation biomarker (MDA) and stimulation of antioxidants enzymes to counteract the active free radicals in *T. evansi* infected camels. Earlier studies demonstrated higher level of MDA in mice infected with *Trypanosoma brucei* (Igbokwe *et al*. 1994) and in camel infected with *T. evansi* (Saleh *et al*. 2009). Superoxide dismutase (SOD) is one of the main

https://doi.org/10.1017/S0031182014001899 Published online by Cambridge University Press

Table 3. Descriptive statistics of the level of cardiac and oxidative stress biomarkers in control and *T. evansi* infected camels

	Control camels ($N = 20$)				Infected camels ($N = 74$)				
Parameter	Mean	Median	Min	Max	Mean	Median	Min	Max	*P*-value*
cTnI (ng mL^{-1})	0·07	0·06	0·00	0·15	1·97	0·81	0·02	6·12	0·0001
CK-MB mass (ng mL^{-1})	0·42	0·36	0·13	1·51	1·08	0·92	0·22	2·40	0·0001
CK (U L^{-1})	134·05	134·76	100·36	159·76	1671·29	1633·25	955·36	2202·47	0·0001
SOD (U mg Hb^{-1})	5·92	6·07	5·11	6·33	3·54	3·55	1·93	9·65	0·0001
sMDA (nmol g $protein^{-1}$)	10·80	10·78	10·23	11·62	21·38	22·45	10·63	24·64	0·0001
eMDA (nmol g Hb^{-1})	109·47	109·36	101·36	113·54	223·13	229·74	110·10	251·36	0·0001

cTnI, cardiac troponin I; CK-MB mass, creatine kinase myocardial band; CK, creatine kinase; eMDA, erythrocytic malondialdehyde; sMDA, serum malondialdehyde; SOD, super oxide dismutase.
* *P*-value resulting from non-parametric Wilcoxon Mann–Whitney test.

Table 4. Descriptive statistics of the level of cardiac and oxidative stress biomarkers in *T. evansi* infected camels with treatment success and failure

	Treatment success ($N = 43$)				Treatment failure ($N = 31$)				
Parameter	Mean	Median	Min	Max	Mean	Median	Min	Max	*P*-value*
cTnI (ng mL^{-1})	0·34	0·12	0·02	1·23	4·23	4·25	0·08	6·12	0·001
CK-MB mass (ng mL^{-1})	0·60	0·42	0·22	1·96	1·74	1·83	0·52	2·40	0·001
CK (U L^{-1})	1427·35	1502·36	955·36	1845·46	2009·67	1982·38	1799·48	2202·47	0·001
SOD (U mg Hb^{-1})	4·08	3·73	3·22	9·65	2·80	2·36	1·93	8·36	0·001
sMDA (nmol g $protein^{-1}$)	21·29	21·67	10·63	24·64	21·51	23·11	10·98	24·36	0·032
eMDA (nmol g Hb^{-1})	225·57	223·14	110·10	251·33	219·76	233·65	110·23	251·36	0·054

cTnI, cardiac troponin I; CK-MB mass, creatine kinase myocardial band; CK, creatine kinase; eMDA, erythrocytic malondialdehyde; sMDA, serum malondialdehyde; SOD, super oxide dismutase.
* *P*-value resulting from non-parametric Wilcoxon Mann–Whitney test.

Table 5. Threshold and test characteristics of selected cardiac biomarkers for prognosis of treatment success and failure in *T. evansi* infected camels

Parameter	Threshold	Sensitivity	Specificity	AUC (95% CI)
cTnI (ng mL^{-1})	3·54	96·77	100·00	0·98 (0·94–1·00)
CK-MB mass (ng mL^{-1})	1·25	83·9	88·5	0·93 (0·88–0·98)

cTnI, cardiac troponin I; CK-MB, creatine kinase myocardial band; AUC, area under the curve.

enzymes incorporated in defence mechanism against reactive oxygen species (ROS). The present study reported lower level of SOD in *T. evansi* infected camel compared to the control. The lower SOD level might be related to its depletion as free radical scavengers during the oxidative process in chronic *T. evansi* infection in camels (Saleh *et al.* 2009). Parallel to the present study, 60% reduction of SOD values in blood of chronically infected patients with *Trypanosoma cruzi* was mentioned (Wen *et al.* 2004). In addition, chronic *T. evansi* in dromedary camels caused obvious decrease in reserve of the antioxidant levels (Saleh *et al.* 2009). In the contrary, Omer *et al.* (2007) reported no change in SOD values in rats with acute *T. evansi* infection.

It was reported that ROS might be implicated in several effects relevant to the pathophysiological mechanisms of chronic heart failure. The superoxide anion is a powerful inactivator of the signalling molecule nitric oxide (NO); the resulting decline in NO bioavailability subsidizes to vascular endothelial dysfunction and the loss of other physiological properties related to inflammation and blood flow. Moreover, the reaction between superoxide and NO creates peroxynitrite, which is itself a strong ROS. Additionally, ROS can moderate the activity of diverse intracellular signalling pathways and molecules with the prospective to trigger specific acute and chronic effects (Grieve and Shah, 2003). The superoxide radical could be challenged with SOD (which significantly decreased in the present study) to convert it into hydrogen peroxides. From another point of view, it was also detected that markers of lipid peroxidation are significantly increased in cases of irreversible myocardial injury (Ansley and Wang, 2013).

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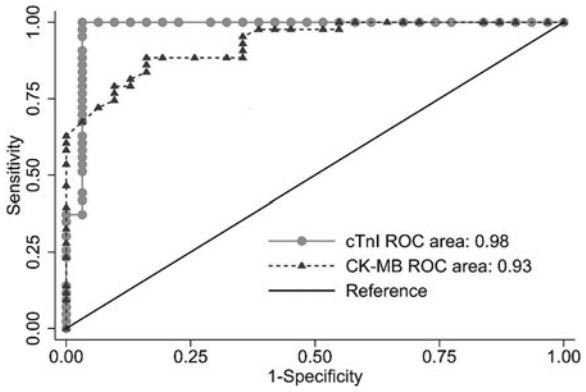

Fig. 1. Receiver operating characteristic plot: comparison of the AUC for cTnI and CK-MB mass.

From the present study, it could be concluded that, elevated serum concentration of cTnI and CK-MB in camels with *T. evansi* infection shed the light on the possible existence of myocardial injury in infected camel. The selected cardiac biomarkers (cTnI and CK-MB) could be used as diagnostic and prognostic biomarkers in camels with trypanosomosis. Antioxidant therapy may assist in the treatment of *T. evansi* infection in camels.

ACKNOWLEDGEMENTS

The authors thank camel owners for their cooperation during sampling procedures.

FINANCIAL SUPPORT

This research was supported by the Deanship of Scientific Research (Project No. 130031), King Faisal University, Saudi Arabia.

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