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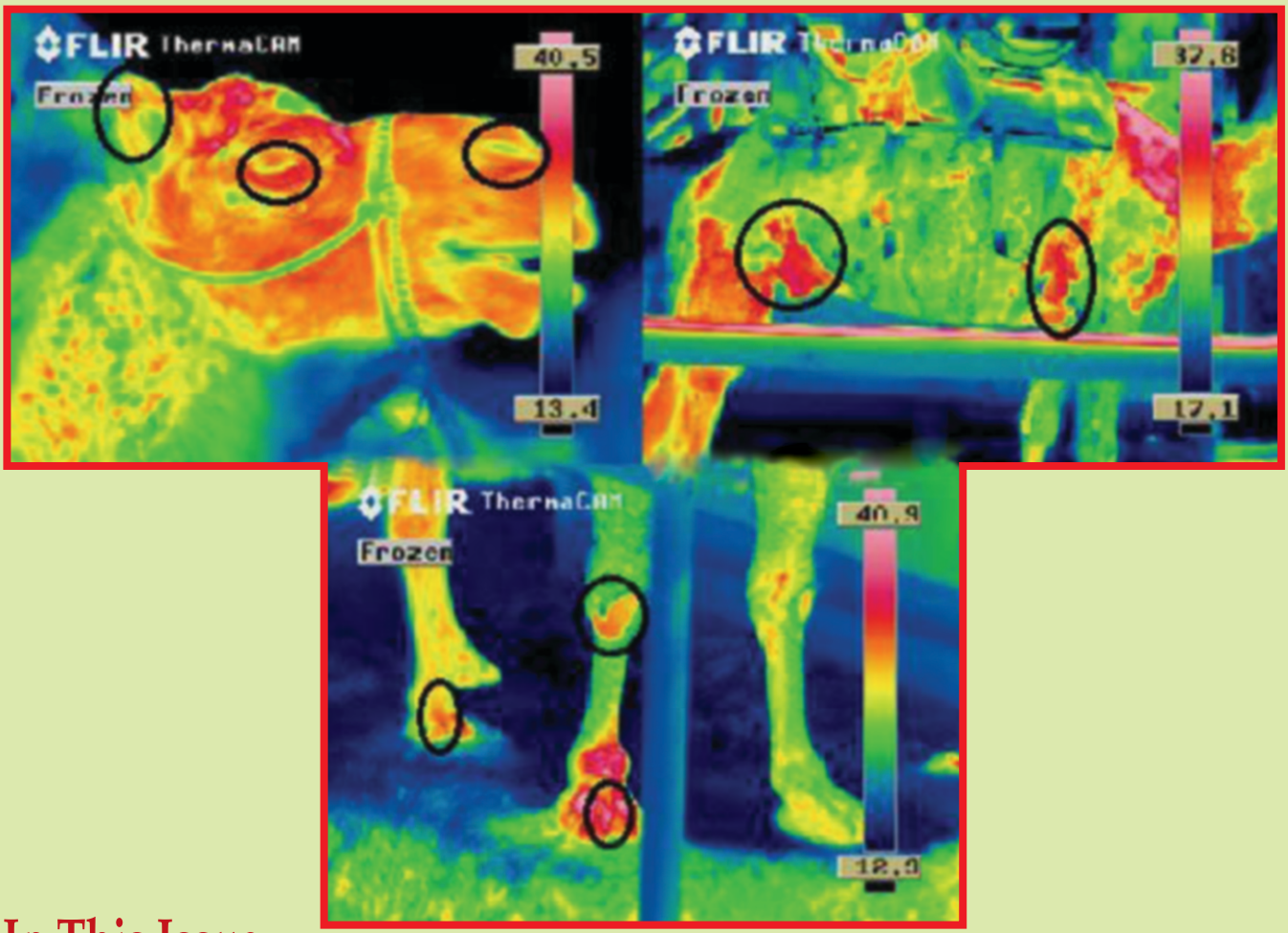

In This Issue

JOURNAL OF CAMEL PRACTICE AND RESEARCH

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Courtesy: Sample images taken during the current study demonstrating lateral view of the head (a), lateral view of the torso (b) and proximal 45° view of the legs with one complete back foot (c). Areas circled in black show the 8 specific areas where surface temperature readings were measured and recorded [Longhorn *et al* (2017). Journal of Camel Practice and Research 24(2):126].

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RESEARCH AND MARKETING OF CAMEL MILK

The changing trends of camel research point to the greater interest of scientists on research and marketing of camel milk. Camel milk is slowly gaining attention of health-conscious people for its nutritional advantages. Camel milk has 10 times more iron and three times more vitamin C than cow's milk. Additionally, camel's milk is lower in cholesterol, fat and high in protein. There have been medical research showing that camel milk has actually helped children with autism live a better life, and individuals who are lactose intolerant do much better. Camelicious stands as the only camel milk brand and dairy production facility in the world to receive EU Commission approval to export to the EU zone. A general report by Euromonitor titled 'Dairy in the UAE' reveals that drinking milk products recorded a value CAGR (Compound Annual Growth Rate) of two per cent in 2016 and are expected to reach sales of Dh1.6 billion in 2021. The current issue has two papers much focused on camel milk, i.e. demonstration of hepatoprotective action of camel milk and a brief review on camel milk proteins, bioactive peptides and casein micelles. Other papers of the current issue include research on serological survey of camel diseases, expression profiles of stress biomarkers, molecular cloning and tissue expression pattern of partial hepatic growth factor cDNA, mastitis and subclinical mastitis, effect of midazolam sedation, effect of energy and protein on draught performance and genetic variations among *Staphylococcus aureus.* The greater interest in parasitology has been observed in the current issue. These include research on *Cystoisospora orlovi*, characterisation of 42kDa antigen in *T. evansi*. Research on relationship between infrared images and heart rate, chlortetracycline in serum and milk, characterisation of methanogenic archaeal community of C1 compartment are also important manuscripts of current issue.

The year 2017 has many activities on camels in form of conferences and festivals. Noteworthy are a special session on Advances in Camel Science in the World Veterinary Congress scheduled at Paris on 5th September and International Camel Conference at Inner Mongolia, China from 22-26 September 2017. The Marwar Camel Culture Festival will be organised at LPPS, Sadri, Rajasthan, India in November this year. Forthcoming year is also important for various camel specific activities which include a Special session on camels with a theme "Camel Research: Challenges and Opportunities" will take place in 11th International Veterinary Congress at Berlin, Germany scheduled on 2-3 July 2018 and International Camel Conference of ISOCARD at Morocco. I am sure that increased frequency of Journal of Camel Practice and Research (Now it is triannual) will reduce the wait period for authors and more number of papers can be published based on camel practice and research.

Passing away of Dr R.O. Ramadan, member of editorial board of JCPR, Professor of Surgery and Radiology, great camel surgeon of Arab world is deeply mourned by the camel scientist community worldover.

(Dr. T.K. Gahlot)
Editor

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SELECTED RESEARCH ON
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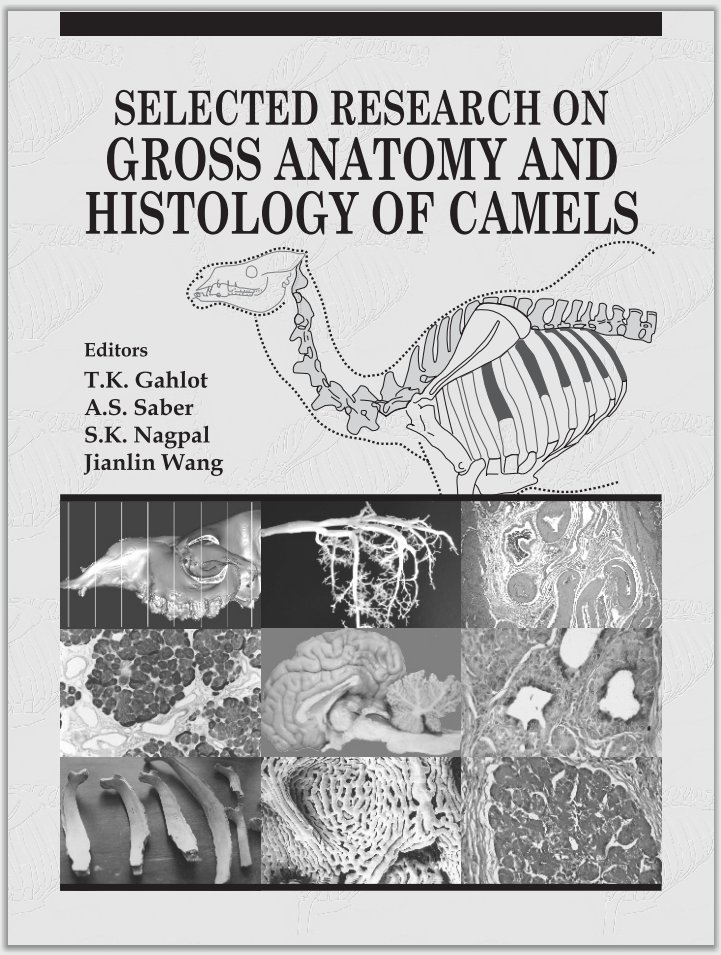
Selected Research on Gross Anatomy and Histology of Camels is a unique reference book on anatomy of dromedary and bactrian camels. This book contains a first ever wide spectrum of histological description of various organs of camels which is depicted by special stains and scanning electronmicroscopy in addition to the gross anatomy, histochemical and immunohistochemical studies. The book has 92 manuscripts in 9 sections, e.g. radiographic anatomy, anatomy of various systems (skeletal, digestive, respiratory, circulatory, urogenital and nervous), common integument and miscellaneous. These manuscripts were published by 158 authors working in 37 laboratories or colleges or institutions from 14 countries in the Journal of Camel Practice and Research between June 1994 to June 2010. Bactrian camel anatomy research was exclusively contributed by the researchers of China. The countries involved in camel anatomy research were China, Egypt, India, Iran, Saudi Arabia, Iraq, Jordan, Japan, Pakistan, Sweden, United Arab Emirates, United States of America, France and Germany. Camel Publishing House has taken a step forward to compile this knowledge in form of a book and this herculian task was accomplished by its dedicated editors, viz. T.K. Gahlot (India), S.K. Nagpal (India), A.S. Saber (Egypt) and Jianlin Wang (China). This classic reference book will serve as a one stop resource for scientific information on gross anatomy and histology of camels.



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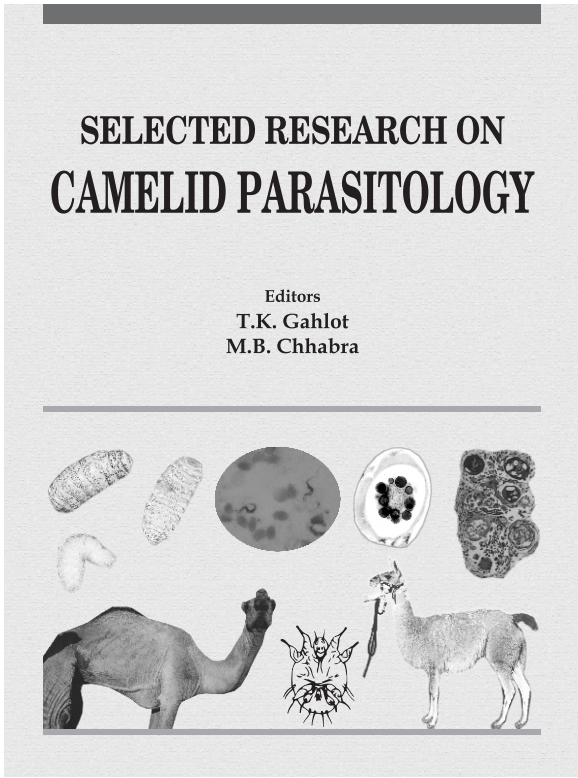

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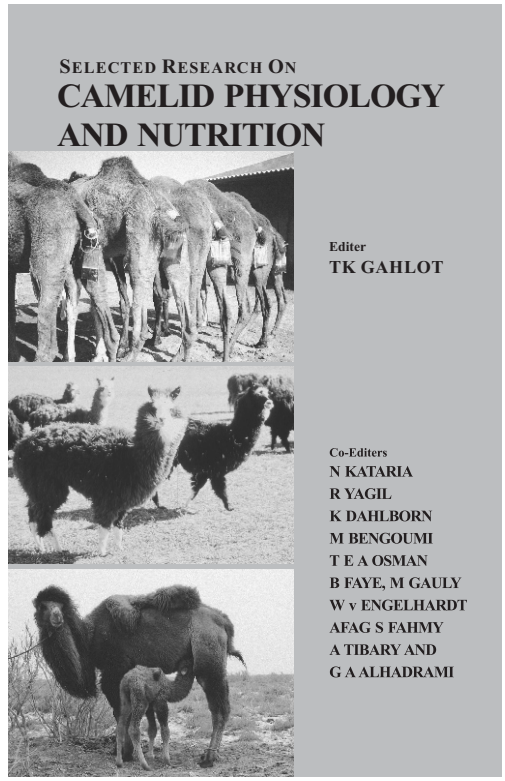

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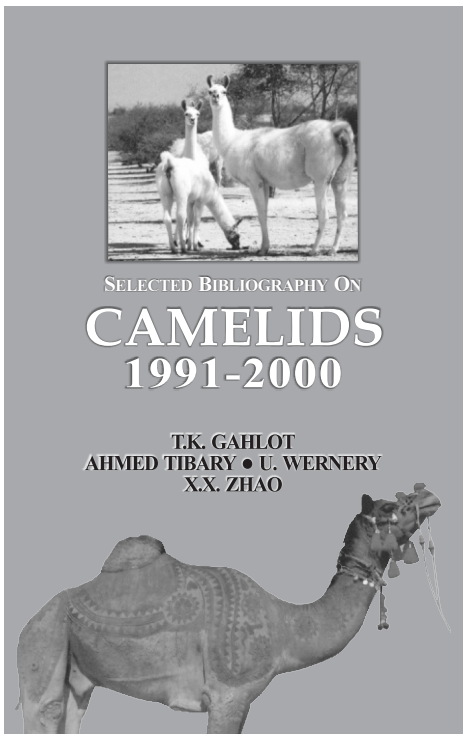

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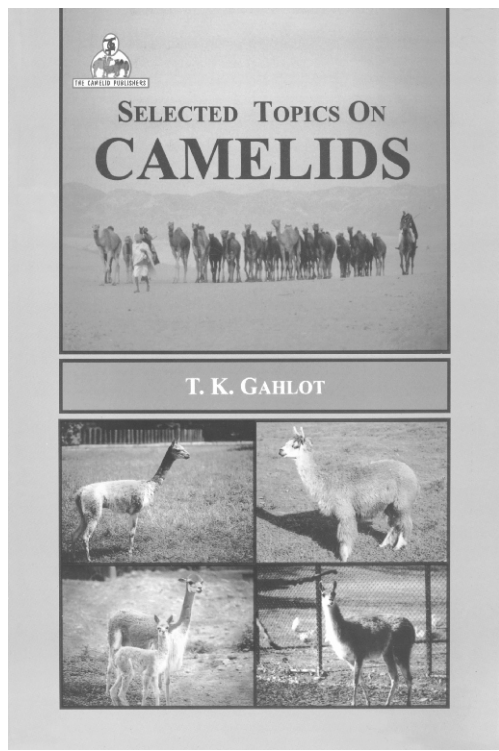

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SEROLOGICAL SURVEY, AN IMPORTANT TOOL FOR CAMEL DISEASE STATUS OF A ZONE OR A COUNTRY

U Wernery
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ABSTRACT

A serological survey was carried out in the United Arab Emirates over the last five years on the dromedary camel population. We followed the OIE Camel Ad hoc group recommendations which was established in 2008 and 2010. This group worked out a comprehensive disease list for Camelids which can be downloaded from the OIE's homepage www.oie.int. The diseases investigated by CVRL follow the OIE listed camel diseases, the results of which are presented here. Our serological survey included 17 infectious diseases divided into viral, bacterial and parasitic diseases. This investigation also addressed the applicability of serological tests for use in camels. This approach was also necessary as many serological tests have never been evaluated for camels and are therefore not mentioned in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals from 2016. Our serological investigations showed three different patterns:

- Dromedaries can get infected by several pathogens exhibiting disease and producing antibodies.
- Dromedaries can get infected by several pathogens producing only antibodies but no disease.
- Pathogens do neither produce disease in dromedaries nor antibodies.

Key words: Serological survey, viral, bacterial and parasitological diseases

Over the last 5 years, a serological survey was conducted on bacterial, viral and parasitic diseases on the dromedary camel population of the United Arab Emirates. The choice of infectious diseases selected for this survey, followed the recommendations of the OIE Camelid Ad hoc group. This group met at the OIE headquarters in Paris in 2008 and 2010 and compiled a comprehensive list of infectious diseases for camelids, which can be downloaded from the OIE homepage: www.oie.int and studied in the 2014 book on camel diseases (Wernery *et al,* 2014). The recommendation of the camelid experts included not only a list of diseases but also proposed to evaluate certain serological tests for use in camelids as most of the tests are not mentioned in the OIE Terrestrial Manual from 2016 because they are not evaluated. First investigations on this subject were already performed by Wernery *et al* (2007 and 2008). The purpose of our serological survey was therefore twofold, firstly to receive data of the health status of the UAE's dromedary population and secondly to investigate the applicability and suitability of serological tests commercially available for use in camelids.

Materials and Methods

In total 17 infectious diseases were investigated of which 6 were bacterial, 8 were viral and 3 were parasitic diseases. The results were compiled in 3 separate tables showing information of test methods used, manufacturer details with email addresses, results of our investigations and in the last column brief explanations were given about the disease status for each infectious disease.

Results and Discussion

Tables 1, 2 and 3 show which infectious diseases were investigated with which results. Four of the 6 bacterial diseases presented in Table 1 produced not only antibodies but also clinical signs. The clinical signs are briefly summarised in the last column of the Table 1. The clinical presentation of Leptospirosis and Anaplasmosis are unknown and there is some doubt if the camel is susceptible to Leptospirosis. However, antibodies against many different serovars have been found (Wernery *et al,* 2014). As there are no clinical signs observed for both diseases so far, the author prefers the term Leptospira and Anaplasma infections instead of "osis".

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In total 8 viral diseases were investigated and as can be seen from Table 2, four of them do not produce a disease in Old World Camels (OWCs) with the exception of Foot-and-Mouth Disease (FMD) in Bactrians (Laska *et al,* 2008). Results of experimental infections and clinical observations from the field confirmed that 2 closely related camel species of Bactrian and dromedary camels possess noticeably different susceptibilities to FMD. Bactrians may contract the disease, dromedaries not (Hohoo *et al,* 2001; Laska *et al,* 2008; Wernery *et al,* 2014). WNF and BVD viruses produce antibodies in camels, but no disease in OWCs, but in New World Camels (NWCs). Rift Valley Fever and Camelpox are the remaining 2 viral diseases which can have a severe clinical impact on camels. Knowledge regarding viral infections in camelids and their economic impact has greatly increased over the years, but there is still a lack of understanding as to whether or not camelids are susceptible to a wide range of important other viral diseases affecting livestock and wildlife.

This lack of knowledge is strikingly associated especially to the Bactrian camels and therefore more research has to be done. This camel conference, the first in China, gives us the opportunity that laboratories working on camel diseases in different countries, come together, cooperate on many subjects as it was proposed by the OIE Camelid Ad hoc group some time ago.

There is not only a lack of knowledge concerning viral diseases but also bacterial diseases. A typical example is Haemorrhagic Septicaemia (HS). The opinion between camel scientists vary widely if HS occurs in camels and therefore a comprehensive study is necessary to clarify the disease complex 'pasteurellosis in camelids' so that diseases with similar clinical pictures, such as anthrax, salmonellosis and endotoxaemia, are less likely to be confused with pasteurellosis. So far bovine *P. multocida* serotypes B:2 and E:2 which produce HS have not been isolated from camels.

Indirect ELISAs (iELISA) are often used for the diagnosis of infections in camelids, but they are firstly not evaluated and lack sensitivity as they include either anti-ruminant or anti-bovine conjugates and not anti-camel. This is important as the Camelidae family possess a unique immune system devoid of light chains (Hamers-Casterman *et al,* 1993).

The ruminant conjugates in commercial test kits possess, however cross-reactivities to camels for example to bovine 73% identity, equine 73% identity, rabbit 66% identity and to mouse only 54% identity. It is therefore obvious that non-species-specific conjugates would require profound studies of indirect ELISAs for camelid diagnostic to minimize false positives and negatives. Ideally, anti-camelid conjugates should replace non-species-specific conjugates. There are several commercial anti-camel conjugates on the market.

Table 1. Test kit details for the serological investigations of antibodies to 6 bacterial diseases, their results and disease status of the UAE camel population.

Bacterial Disease	Test Details	Manufacturer	Email	Results			
				Total sera	Positive	%	Disease Status
Tuberculosis Tb	Lateral Flow	Diagnostic Systems Inc. USA	info@chembio.com	1.607	7	0.4	M. bovis and other biovars produce disease
Brucellosis	RBT	Vircell - Spain	info@vircell.com	26.375	1324	5	Very common mainly *B. melitensis*
Anaplasma Infection (Rickettsiales)	Competitive ELISA *A. marginale,* ovis, centrale	VMRD, USA	order@vmrd.com	1.713	9	0.5	Subclinical, no disease
Leptospira Infection	Microscopic Agglutination Test (MAT)	You keep your own strains alive in lab.	Weybridge, UK	212	21	12	*L. copenhageni, L. ballum, L. autumnalis, L. tarassovi, L. javanica,* no disease
Caseous Lymphadenitis (CLA)	Indirect ELISA CVRL	Inhouse indirect ELISA with LPD	CVRL DUBAI in-house cvrl@cvrl.ae	481	48	36	Caseous Lymphadenitis very common in China, Mongolia, East Africa also with internal abscesses
Para-tuberculosis	Indirect ELISA CVRL	Inhouse indirect ELISA with MAP (Purified extract)	CVRL DUBAI in-house cvrl@cvrl.ae	381	6	1.6	Very common, devastating disease in Saudi Arabia and other countries, untreatable.

Table 2. Test kits details for the serological investigations of antibodies to 8 viral diseases, their results and disease status of the UAE camel population.

Viral Disease	Test Details	Manufacturer	Email	Results			
				Total sera	Positive %		Disease Status
FMD	cELISA	Cedi Diagnostics B.V.	cedidiagnostics@wur.nl	1.631	0	0	Dromedaries resistant, Bactrians not
PPR	cELISA	Biological Diagnostic Supplies Ltd. (BDSL)	mail@bdsl.uk.com	1.910	0	0	Questionable, infection trial negative
WNF	cELISA	ID Vet	idvet.info@id-vet.com	1.612	41	2.5	No disease but infection virus isolated, lineage 1a
RVF	cELISA	Biological Diagnostic Supplies Ltd. (BDSL)	mail@bdsl.uk.com	1.120	8	0.7	Severe disease, abortion some imported camels are positive
BT	cELISA	ID Vet France	idvet.info@id-vet.com	1.703	358	21	No disease, no virus isolated, 28 serotypes
Camelpox	SNT	CVRL	inhouse cvrl@cvrl.ae	2.340	2000	85	External and internal pox lesions, not in Australia
BVD	cELISA	Institut Pourquier France	info@institut-pourquier.fr	1.210	19	1.6	Questionable, but severe disease in NWCs
EBL	cELISA	Institut Pourquier France	info@institut-pourquier.fr	1.200	0	0	No disease

Table 3. Test kits details for the serological investigations of antibodies to 3 parasitological diseases, their results and disease status of the UAE camel population.

Parasitic Disease	Test Details	Manufacturer	Email	Results			
				Total sera	Positive %		Disease Status
Surra *T. evansi*	iELISA	In house CVRL	cvrl@cvrl.ae	45.567	1.530	1.5	Very common, anaemia sudden death not in Australia
Toxoplasmosis	Direct Agglutination	Bio-Mérieux France	biomerieux.com	1119	48	40	No disease, but high sero-prevalance
Neosporosis	cELISA	VMRD, Inc.	vmrd@vmrd.com	1119	157	14	Old World Camels no disease, NWCs abortion

Three parasitic diseases were serologically investigated of which Surra is the most prevalent one. It occurs in all camel rearing countries except Australia and is mainly caused by *Trypanosoma evansi*.

Toxoplasma and Neospora parasites produce antibodies in camelids but no disease in OWCs has been observed.

Over the last two decades our knowledge on infections in the camel family has greatly increased by field or laboratory investigations, but there is still a lack in different fields. This conference gives us the opportunity to narrow this gap.

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Cystoisospora orlovi (EIMERIORINA: SARCOCYSTIDAE) – A LITTLE KNOWN COCCIDIAN OF THE OLD WORLD CAMELIDS

Rolf Karl Schuster[1], Saritha Sivakumar[1], Peter Nagy[2], Jutka Juhasz[2], Ahmed Ismail[2] and Jörg Kinne[1]

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ABSTRACT

Based on the morphology of its oocysts *Cystoisospora orlovi* was described in camel calves in Kazakhstan in 1950. Over decades *C. orlovi* was considered a pseudo-parasite until alterations caused by this parasite in the colon of dromedary calves were described. During an observation period of 150 months (January 2005 – June 2017), 85 cases of coccidiosis due to *C. orlovi* were observed in 72 camel calves and 13 adult dromedaries in Dubai (United Arab Emirates). Most of the cases in camel calves were seen between the months of December to May in an age group of up to 35 days while the majority of cases in adult camels occurred in summer. Contrary to *Eimeria* infections in camelids, *C. orlovi* causes a diphtheroid colitis with multiple erosions of the mucosa of the large intestine. Most of the cases were diagnosed post mortem. Only 11 camels in which the parasite was diagnosed *intra vitam* survived. The sporadic occurrence of cystoisosporosis in single suckling calves suggests a vertical transmission of the parasite from mother to calf.

Key words: *Cystoisospora orlovi*, coccidia, dromedary, Dubai, United Arab Emirates

During a survey on camel coccideans in the Almaty district of Kazakhstan, Cygankov[1] (1950) discovered a previously unknown species for the camel with oocysts containing two sporocysts. The new coccidian was named *Isospora orlovi*. It was found in 10 out of 19 camel calves in an age between 10 to 35 days. The validity of *I. orlovi* was doubted by protozoologists (Pellerdy, 1965; Kheysin, 1972; Levine, 1985) who regarded it as pseudo-parasite[2] of camels. A further finding of *Isospora* sp. in camels morphologically matching *I. orlovi* was made in India by Raisinghani *et al* (1987). *Isospora* oocysts described in camels in Syria by Dariush and Golemanskij (1993) had other dimensions and were, due to the presence of Stieda bodies, attributed to spurious parasites (Duszynski *et al,* 2001).

Kinne *et al* (2001) described for the first time alterations in the colon of camel calves in Dubai (UAE) caused by *Isospora* sp. suggesting that this parasite was identical with *I. orlovi*. Based on these findings the material was used for a redescription of *I. orlovi* (Kinne *et al,* 2002). First cases of *I. orlovi* in camel calves from Rift Valley Province in Kenya were described by Younnan *et al* (2002). Further cases were reported from the same locality by Bornstein *et al* (2008).

Morrison *et al* (2004) sequenced the 18s rRNA subunit and placed *I. orlovi* in one clade with the human pathogen *I. belli* in direct neighborhood with *I. suis* and *I. felis*. Based on morphological peculiarities and supported by molecular examinations the paraphyletic genus *Isospora* was split into 2 monophyletic genera: *Isospora* found in birds and *Cystoisospora* that infect mammalian hosts (Barta *et al,* 2005).

The aim of this paper is to summarise our experience with *Cystoisospora orlovi* in dromedaries in the UAE obtained over a 12½ year's period.

Materials and Methods

A total of 76,969 and 2,885 faecal samples of adult dromedaries and camel calves, respectively were examined at the Central Veterinary Research Laboratory (CVRL) in Dubai during a time period of 150 months (January 2005-June 2016). While the

1. There are several versions of spelling of the authors name: Zigankoff (Pellerdy, 1965), Tsygankov (Levine, 1985) but the right transcription from the Russian original reference would be Cigankov.

2. A pseudo-parasite or spurious parasite is a development stage of an apparent parasite that entered the alimentary tract by food or water and is passed with faeces.

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majority of these samples were sent for a routine parasitological checkup, 2,084 originated from necropsied camels (996 adults and 1,088 calves).

During the routine necropsy, organ samples including pieces of the large and small colons were taken and fixed in 10% neutral formalin, dehydrated through graded alcohols and embedded in paraffin wax. Sections (3 µm) were cut and stained with haematoxylin and eosin (HE) and Periodic acid schiff (PAS) and examined under the microscope (Olympus BX 53).

Faecal samples or content of the colon taken per rectum were used for parasitological examination. Faecal samples were processed with the flotation method in centrifugation tubes using saturated $NaCl/ZnCl_2$ solution (density 1.24). Carbol fuchsin stained faecal smears (Potters and van Esbroeck, 2010) from suckling calves and diarrhoeic samples were examined in addition to exclude *Cryptosporidium* oocysts. In addition, scrapings from colon mucosa were examined by direct microscopy.

For morphometric studies, 10 positive faecal samples were suspended in a 2.4% aquatic potassium dichromate solution ad kept in petri dishes for one week at 25°C. Ten sporulated oocysts were microscopically measured from each sample. For this, one ml of the faecal suspension was then mixed with 12 ml of a concentrated $ZnCl_2$/ NaCl solution in a plastic tube covered by a cover slip. After 5 min, the coverslip was removed and placed on a glass slide for microscopical examination. Measurements were taken with a microscope (Olympus BX 51) connected via a camera (Olympus DP 27) to a computer operated by the software "cellSense Dimension". The length and width of 100 oocysts and containing sporocysts were measured. In addition, 10 unsporulated oocysts freshly harvested from colon mucosa were also measured.

In order to establish the source of infection, the mother and all calves of the group that were in contact with 3 positive individuals were coproscopically checked in 2008, 2014 and 2016.

On two separate occasions, two adult female dromedaries were orally infected with 200,000 and 1,000,000 sporulated oocysts in 2010 and 2017, respectively. For a period of 3 weeks, faecal samples of these camels were examined daily for the presence of *Cystoisospora oocysts*.

Results and Discussion

During the 150 months' observation period *C. orlovi* was detected in faecal samples of 72 camel calves and 13 adult camels (Table 1). These samples, originated from 70 carcasses (64 calves, 6 adult camels) sent for necropsy and 15 from live camels (8 camel calves, 7 adult camels) suffering from diarrhoea. All calves were born in Dubai; of the 13 adult camels, 7 originated from the UAE, 6 others were imported from Saudi Arabia and Sudan. The majority of positive samples (n = 64) originated from The Emirates Industry for Camel

Table 1. Frequency of *Cystoisospora orlovi* positive samples in camel calves and adult camels in Dubai between January 2005 and May 2017.

Year	Camel calves				Adult camels			
	Examined	Positives			Examined	Positives		
		Total	EICMP	Other farms		Total	EICMP	Other farms
2005	25	0	0	0	5,540	0	0	0
2006	578	3	0	3	11,702	0	0	0
2007	399	3	1	2	11,301	0	0	0
2008	403	6	1	5	12,223	3	1	2
2009	86	4	1	3	11,474	0	0	0
2010	121	12	9	3	3,212	9	9	0
2011	191	13	12	1	3,174	0	0	0
2012	125	4	3	1	3,964	1	1	0
2013	114	0	0	0	2,718	0	0	0
2014	503	5	5	0	4,258	0	0	0
2015	145	4	3	1	3,182	0	0	0
2016	124	12	12	0	3,900	0	0	0
2017	71	6	6	0	321	0	0	0
2005-2016	2,885	72	53	19	76,969	13	11	2

Table 2. Distribution of *C. orlovi* in camel calves and adults throughout seasons of the year.

Age group	Month											
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Calves	17	17	16	7	5	1	2	0	0	1	1	5
Adults	1	0	0	1	0	3	6	0	2	0	0	0

Milk Products (EICMP), a large scale camel dairy farm that started operation in 2006 and has currently, a camel population of 4,500 animals. The remaining 21 positive samples came from camels of traditional breeding farms in Dubai. *Cystoisospora* cases were unequally distributed throughout the observation period. As seen from table 1, the parasite was not diagnosed in 2005 and 2013 while in 2010, 2011 and 2016, 21, 13 and 12 positive cases were seen. Most positive cases in calves occurred during winter and spring between December and May while in 11 out of 13 adult camels the parasite was diagnosed in June (n=3), July (n=6) and September (n=2) (Table 2).

In an early stage of the infection examination of faecal samples gives a negative result but unsporulated oocysts and zygotes can be seen in direct microscopy of mucosal scrapings (Fig 1). In the majority of cases however, oocysts were already in an advanced stage of sporulation. Due to the thin (1 µm), smooth, two-layered oocysts wall, the shape of the oocysts (ellipsoid, ovoid or 8-shaped) was determined by the configuration of the 2 sporocysts within the oocyst (Fig 2, 3). Sporulated oocysts had an average length of 31.3 (range: 27.0-34.8) µm and a width of 20.8 (range: 17.8-24.4) µm. Micropyle, polar granule and oocyst residuum were absent. The ellipsoidal shaped sporocysts were 18.4 (range: 15.0-21.9) µm long and 14.9 (range: 12.6-19.6) µm wide. Stieda, substieda and parastieda bodies were absent. The subsequent length/width indices for oocysts and sporocysts were 1:1.5 and 1:1.1, respectively. Sporocysts contained 4 elongated, banana shaped sporozoits (12-15 x 4.5-5 µm) with rounded ends and a sporocyst residuum formed by cluster of small granules. Most of the unsporulated oocysts were oval in shape. Their average length and width was 28.6 (range: 25.4-30.2) and 20.5 (range: 19.4-21.5) µm, respectively.

Most cases of cystoisosporosis were diagnosed post mortem. Only 6 calves and 5 adults from EICMP farm in which the parasite was diagnosed *intra vitam* survived. The fate of 2 adult camels and 1 calf from other Dubai farms could not been followed further.

Alterations caused by *C. orlovi* were concentrated in the colon that contained crumbly yellowish to grey content (Fig 4). A diphteroid to haemorrhagic colitis with multiple erosions in the mucosa (Fig 5) is pathognomonic for *C. orlovi* infections. At the end of the prepatent period, micro- and macrogamonts can be spotted in histological sections of the colon (Fig 6). During the patent period histocuts contain a large amount of sporulated and to a lesser extent unsporulated oocysts (Fig 7).

For 48 camel calves born on the EICMP farm, the exact age was submitted with the requisition form (Table 3). Thus, in 85.5% *C. orlovi* was diagnosed in calves at an age of up to 35 d. The youngest calf

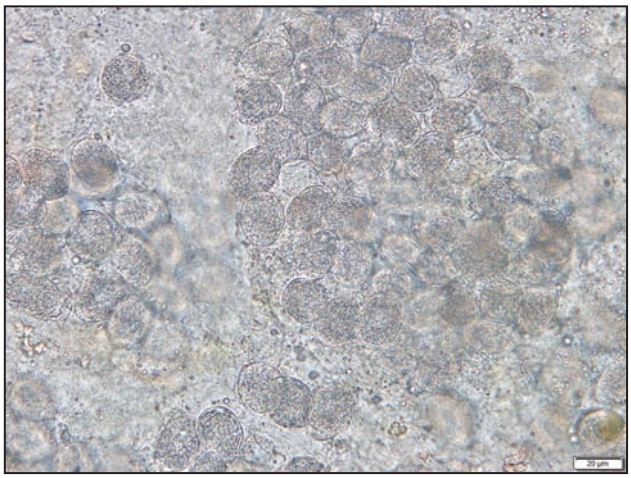

Fig 1. Unsporulated oocysts in a mucosal scraping from small colon.

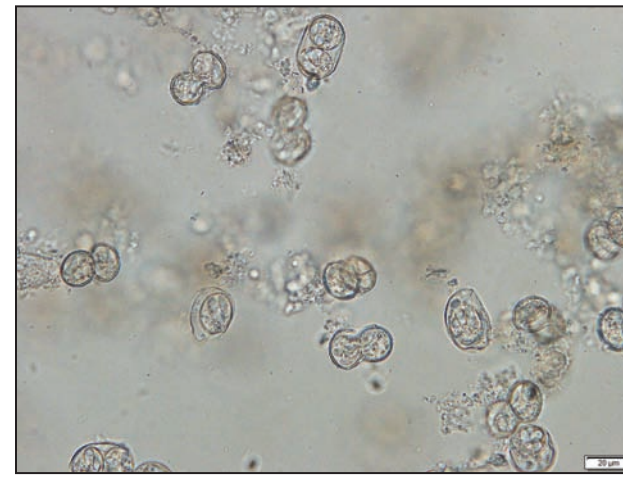

Fig 2. Sporulated *C. orlovi* oocysts. Oocyst contain 2 ellipsoid sporocysts the configuration of which determines the shape of the oocyst.

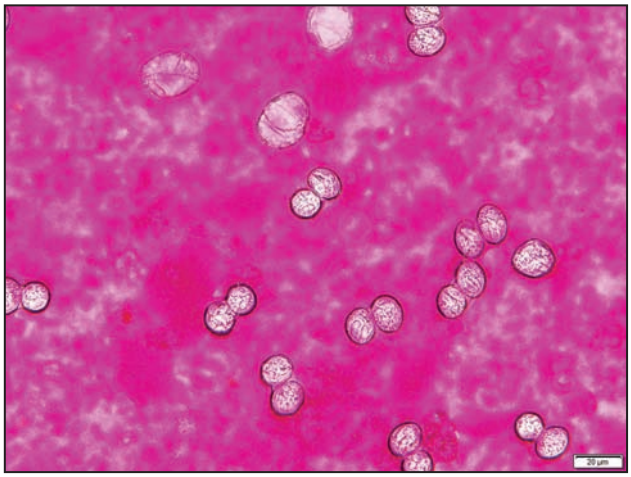

Fig 3. Sporulated and unsporulated *C. orlovi* oocysts in a fresh carbol fuchsin stained faecal smear.

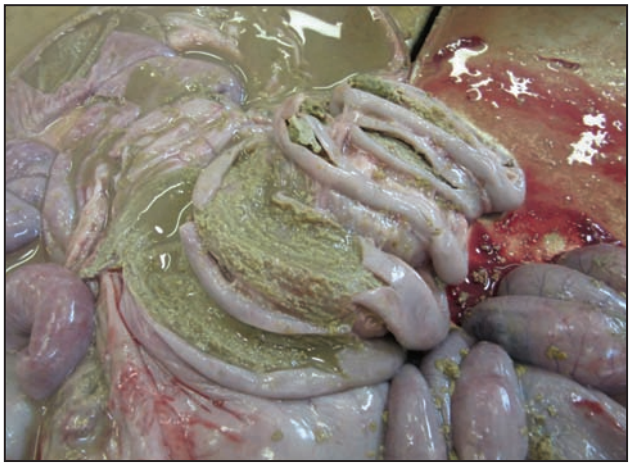

Fig 4. The small colon of a *C. orlovi* infected camel calf with crumbly grey content.

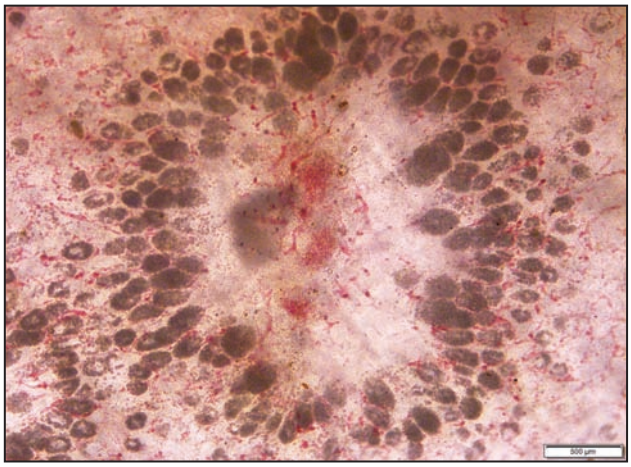

Fig 5. Mucosa of the colon with erosions and bleedings is pathognomonic for *C. orlovi* infections.

diagnosed with a *Cystoisospora* infection was 9 d old while the oldest calves were 115, and 150 d old. The age of the adult camels was between 4 and 8 years. It is noteworthy that all 5 fatal cases in adult camels

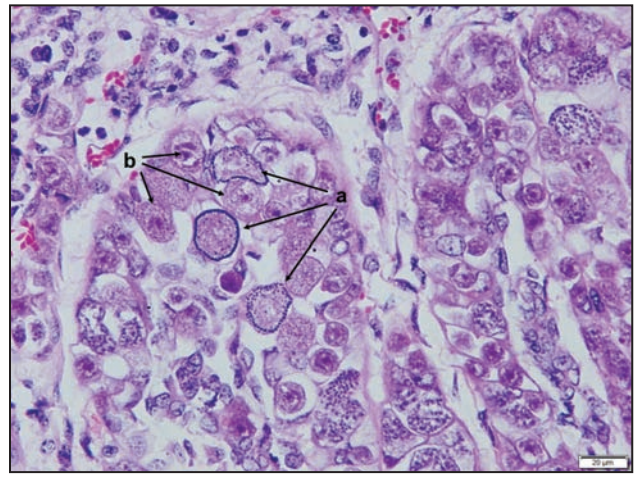

Fig 6. Histological sections of the small colon with microgamonts (a) and macrogamonts (b).

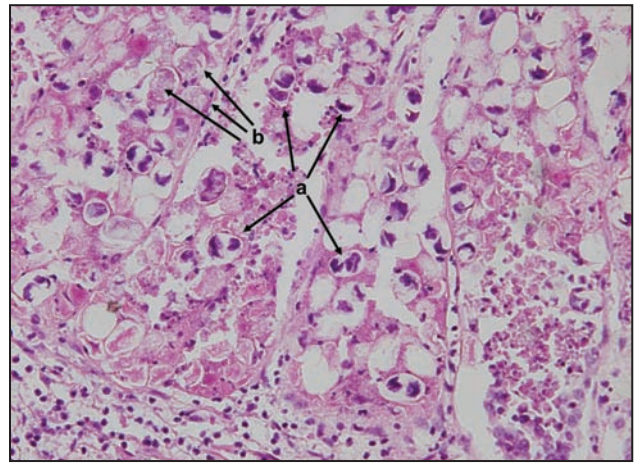

Fig 7. Histological sections of the small colon of a camel calf that died with a patent infection. Multiple sporulated (a) and unsporulated (b) oocysts can be seen between the villi.

in 2010 had severe concurrent bacterial infections (*Rhodococcus, Corynebacterium, Mycoplasma*) or suffered from clostridial enteroxemia.

Table 3. Age of *Cystoisospora orlovi* infected camel calves.

Age in days	Number of positives	
	n	in (%)
< 14	1	2.1
14 - 21	13	27.1
22 - 28	18	37.5
29 - 35	9	18.8
36 - 42	2	4.2
43 - 49	1	2.1

Coproscopical examination of the mothers and contact calves of three *Cystoisospora* positive calves gave negative results. None of these 25 samples revealed the presence of *Cystoisospora oocysts*. Also, the oral inoculation of 2 adult female camels with

sporulated *Cystoisospora oocysts* did not result in shedding of oocysts during the observation period of 21 days.

In the first description of *C. orlovi* by Cygankov (1950) the host species Bactrian or dromedary camel was not mentioned. Both species of Old World camels are kept in Kazakhstan. All later findings in India, UAE and Kenya however, were made in dromedaries.

In our material, most cases occurred during the main calving period in winter and spring (December to May) mainly in calves at an age between 2 and 5 weeks with a history of diarrhoea. Kinne *et al* (2001) reported 8 fatal cases of camel cystoisosporosis occurring between January and March 2001 in traditional camel farms in Dubai. The age of the calves was estimated to be 4 to 8 weeks. Similar findings were made in Kenya (Bornstein *et al,* 2008), where the parasite was diagnosed in 19 to 30 day old camel calves in ranched herds. The age of infected calves in pastoral herds in Kenya was 20 to 56 days. The excretion of *C. orlovi* oocysts is not compulsory restricted to young calves since we saw the parasite also in single calves at an age of up to 5 months. Also, the only camel calf from India that was diagnosed with *C. orlovi* was 6 months old (Raisinghani *et al* (1987) and our material included findings of *Cystoisospora* oocysts also in 13 adult camels.

Contrary to *Eimeria* spp. *C. orlovi* occurs sporadically mainly in young suckling calves and was, surprisingly detected so far only in 4 countries. Despite several attempts, the source of infection was not determined. A horizontal transmission as known for *Eimeria* infections can be excluded since contact calves and the calf mothers were coproscopically negative for *C. orlovi* oocysts.

In *Cystoisospora* species of carnivores extra-intestinal monozoic cysts occur in lymph nodes, liver, spleen or muscles of paratenic (rodents and farm animals) as well as of definitive hosts and play a role in the life cycle of these coccidians.

The porcine *C. suis*, the best investigated representative of the genus, has a direct life cycle (Harleman and Meyer, 1983). The two-peaked excretion of oocysts after a single experimental infection however, suggested the existence of extra-intestinal development stages in piglets. So far, these stages have not been visualised. Still, gnotobiotic piglets shed oocysts after intraperitoneal inoculation of tissue homogenates from experimentally infected piglets (Shrestha *et al,* 2015). Extra-intestinal stages were also described in the human pathogen, *C. belli* (Lindsay *et al,* 2014). The close genetical relation between *C. belly* and *C. orlovi* (Morrison *et al,* 2004) suggested the existence of extra-intestinal stages also in *C. orlovi*.

In our opinion, it must be considered that the transmission of *C. orlovi* is vertically from mother to calf, probably via milk and, that an ingestion of *C. orlovi* oocysts by adult camels does not lead to a patent infection but most probably leads to a dissemination of sporozoites in extra-intestinal tissues. Stress situations like calving could activate these stages. Rare cases of cystoisosporosis in adult camels during the hot summer months might be attributed to heat stress or to the depression of the immunity due to other conditions as it was the case in 5 adult camels in 2010.

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ESTABLISHING THE RELATIONSHIP BETWEEN INFRARED IMAGES AND HEART RATE AS A POTENTIAL INDICATOR OF METABOLIC ACTIVITY IN THE DROMEDARY CAMEL (*Camelus dromedarius*)

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ABSTRACT

The goal of the present research is to provide a simpler method of measuring metabolic activity in the dromedary camel and to determine the major route of heat dissipation within this species. Eight dromedary camels were equipped with heart rate monitors on moderately active and low activity days. Thermal images, heart rate readings, ambient air temperature, humidity, wind speed and the presence of direct solar radiation were recorded. The results of a random regression demonstrated that, of the areas observed *via* thermal image, the surface temperature of the inner thigh was the most closely associated with heart rate. The inner thigh and lower abdomen were also noted as being the largest continuous areas of heat loss based on infrared images, while the axillary region demonstrated the greatest increase in heat dissipation as activity and ambient air temperature increased. These results exhibit positive evidence for further research into the use of infrared images as predictors of metabolic activity in the dromedary camel.

Key words: Dromedary camel, heart rate, infrared imaging, metabolic activity

A direct measure of heart rate (HR) can be used to estimate the level of metabolic activity is an indirect indicator of the oxygen demand of the body and changes in direct correlation with activity level (Green, 2011; Schmidt-Nielsen *et al*, 1967; Wilson *et al*, 2006). Use of the heart rate estimate metabolic activity in free-ranging homeothermic vertebrates has been termed "the heart rate method" and is a relatively popular tool for examining non-domesticated species, or species too large for the use of direct calorimetry to be feasible (Green, 2011).

Montanholi *et al* (2008), Mader *et al* (2009) and Huntington *et al* (2012) have established that the use of infrared images can replace the need for calorimeters and other constrictive equipment for determining feed efficiency in cattle. This recently reported relationship has the potential to significantly reduce costs of feeding trials and could simplify the manner in which captive exotic animals in zoological parks are studied. The present study was aimed to simplify the methods for determining metabolic activity by investigating whether or not infrared images can be effectively used to predict heart rate in dromedary camels. Additionally, this research was aimed to determine localised areas of heat dissipation that occurs with an increase in physical activity.

Materials and Methods

Experimental Methods

Experimental procedure aimed to correlate the heart rate of a camel with the surface temperature of a given body part as a potential indicator of metabolic activity. Thermal images were taken of different focus areas on the camels, each of which were simultaneously equipped with a heart rate monitor. Camels were examined during both low activity and moderate activity days. A low activity day was deemed to be when the camels were free to move about their enclosure at leisure and was used as a control. A moderate activity day was deemed to be when the camels were involved in giving camel rides to the public and walked a set amount each day.

Data collection was conducted at both the Bowmanville Zoological Park (BZP) and the Toronto Zoo (TZ), with observations taking place during the daylight hours of late morning to late afternoon. Data collection occurred for 2 weeks during the beginning of May and 2 weeks starting in mid-June, when the exposure to solar radiation and increasing

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daily air temperature was sufficiently high enough to demand an increase in the need for thermoregulatory mechanisms.

Data collections occurred for a maximum of 5 hours during the hours of most direct sunlight exposure and only when BZP and TZ were open to the public. All observations and images were recorded from outside the enclosures or at a distance of at least 1.8 meters from the animal during trials that involved rides. This distance was established by the BZP camel handlers as a safe distance.

Initial observation days occurred before the commencement of the experimental procedure to determine which areas of the body appeared to lose the greatest amount of heat, as there is no record of previous thermal imaging analysis on dromedary camels. During the initial observation days, camels were inside the barn and at rest. Based on the analysis of the initial thermal images, 7 localised areas were deemed appropriate for further experimentation as they demonstrated the highest and most consistent areas of heat loss: the eye, ear, nostril, front feet and back feet between the 1st and 2nd digits, the inner thigh surrounding the urethra and the axilla. A few other areas were noted as demonstrating comparative heat dissipation, however, in order for this research to be repeated, only clearly defined areas that could be consistently imaged were chosen.

All the animals used for data collection were dromedary camels (*Camelus dromedarius*) belonging to BZP and trained in a free contact environment. A total of 8 camels, ranging from 3 to 19 years of age, were used for data collection. Camels were observed either on moderately active or low activity days. Low activity days occurred in combination at BZP and TZ depending on the locations of the camels to be used for experimentation each day. Moderately active days, where the camels provided rides to the public through a set course, were conducted solely at TZ. With the exception of one individual, all camels were male and at a good fitness level as per veterinary examination (Table 1). Two males included for data collection on low activity days did not have corresponding moderate activity data days, simply due to their availability and need at other locations for rides. As such, while no moderate activity data was collected for these males, they were of comparable body fitness score to the other males involved throughout the course of experimentation based on age, weight, height and veterinary examination. The youngest male was deemed by the keepers too young to be involved with camel rides with a rider; as such his moderate activity involved walking the ride circuit without a rider, but at the same number of circuits to those camels with a rider.

All moderate activity data was obtained out of doors where the animals were exposed to the elements, whereas 5 out of 8 low activity days were completed out of doors with the remaining three were performed inside the barn with all barn doors open and with at least one other camel present with the individual being observed to limit their anxiety while removed from the herd.

Dromedary camels under observation were equipped with equine Inzone heart rate monitor attached using tensor bandages and medical tape. Tape was necessary to place the appropriate pressure on the negative electrode lower on the chest due to the characteristic beveling of the camel rib cage where it meets the cush pad. A small amount of rubbing alcohol was used to wet the surface of the electrodes and dermal layer of the camel, then a measure was taken with the receiving watch (heart rate was displayed without decimal places as an integer in beats/minute). This watch had to be within a foot of the transmitter in order to receive a heart rate reading. As all individuals had previously been used for rides and were accustomed to saddle straps, one hour was deemed sufficient time for acclimation to the heart rate monitor prior to commencing experimental procedure. There were no problems with the monitors on low activity days; however, on moderate activity days when the monitors were under the saddle straps the rides had to be stopped on several occasions to allow for monitor readjustment.

Once experimental procedures began, a baseline heart rate was obtained for all dromedary camels. During low activity days, heart rate measurements were taken every 15 minutes with simultaneous thermal images, ambient air temperature, humidity, wind speed and solar radiation measurements. For comparison, data was collected every 6th lap the camel completed around the ride area on moderate activity days, as this was estimated to be within approximately +/- 2 minutes of the 15 minute mark. Thermal images were taken using a ThermaCAM SC 2000 thermal imaging camera (FLIR systems, Danderyd, Sweden), with a built-in lens 24°. This camera detected naturally emitted longwave radiation (7.5-13μ) from the animal's surface and converted it into electrical signals which were then processed into thermal images. Data collection consisted of a set of 3 images: lateral image of the head, the torso and the frontal limbs with at least one front foot and back foot

in a single image (Fig 2). Ambient air temperature (°C) and humidity (%) were measured using the Kestrel 4000 (Nielsen-Kellerman, Boothwyn, PA) and a Sims DIC-3 Anemometer (Simer1 Instruments, Annapolis, MD) measured the wind speed (m/sec). Whether the camel was in the sun or share, or whether the sky was overcast, was recorded through the experimental procedure.

In addition, thermal images were taken wherever possible, while the animal urinated and the time recorded. These images may provide a reasonable measure of the animals' internal body temperature (Benedict and Lee, 1936) and be used as a comparison to the surface temperatures.

ThermaCAM Explorer 99 and ThermaCAM Researcher Professional Software were used to analyse all thermal images taken. As this software allows for localised analyses of the images taken, it was possible to focus solely on the surface temperatures for the areas of interest (Fig 2). The warmest temperature present for each focus area was then recorded with a reference as to whether or not the area was in direct sunlight. This was done to take into account any unusually high temperature that was not the result of dissipating metabolic heat alone, but due to direct absorption of solar radiation. All surface temperature were recorded in degrees Celsius.

Statistical methods

All statistical procedures were performed using SAS statistical software. A hierarchical linear model was used to determine if there was a correlation between the surface temperature of body parts and heart rate. Measurements of heart rate over time on the camels were analysed as a repeated measures model using the mixed procedure (SAS 9.3) which included camel as a random effect in order to account for differences in average heart rate among the camels. Both activity level (moderate *versus* low) and weather conditions (sunny *versus* overcast) and their interaction were included as fixed effects and air temperature and humidity were included as covariates. Wind speed was negated as an effect because there were no instances in which the wind speed reached or was above 4m/s. This is known to be the minimum speed required to cause an impact upon and animal's surface temperature (Tregear, 1965). The initial model included temperature measurements and humidity, eye, front and back foot, ear, axilla and thigh on each camel as predictors and the model was re-run repeatedly in a backwards stepwise fashion, eliminating the predictor with the highest p-value until any remaining predictors were significant at the 20% level (P<.20).

Thermal images that were taken while the camels urinated were not consistently obtained as such no statistical analyses were performed on that portion of the data.

Results

Average heart rates analysed according to individual, when compared between Low Activity (LA) and Moderate Activity (MA) days, were shown to be significantly higher on the MA days (Student's paired t-test, p=0.0058 to p=1.3047 x 10^{-8}), for those camels that participated on both MA and LA days, with average heart rates increasing by 12, 16, 21 and 35% (Table 1).

The surface temperatures for all 7 body areas under observations were plotted on a standard lines regression where the resulting ANOVA demonstrated the highest p-value for the ear (Table 2). The corresponding ear data was the eliminated and the model rerun. The back foot was the second set of data removed, followed by the eye, front foot, nostril and finally axilla in the subsequent models. In all cases it was evident that the thigh showed the closest association to the linear regression model (Table 3).

The final model, without any random regressions, included a regression on the mean value of all the slopes on thigh temperature (p<0.0001), along with activity level (p<0.0001), sun exposure (p=0.2572), activity level and sun exposure interaction (p=0.0788), ambient air temperature (p=0.0004) and relative humidity (p=0.2700) as fixed effects and camel as a random effect. The Akaike criterion for this model was 1563.3. This model was then rerun but with the addition of random regressions on thigh temperature for each camel results in an Akaike criterion of 1542.2.

Overall, heart rate was 5.9 bpm higher when the camel was in the sun (p<<0.0001) and 0.6 bpm lower when it was overcast, although this latter value was not found to be statistically significant (p=0.5112). The average slope changed by -0.46 bpm/°C when the ambient air temperature was higher (p=0.0002) and a slope change of -0.077 was estimated during high humidity (p=0.0066). The mean of the random intercepts was 43.5976 and the random slopes on thigh were 0.4058 for the group. This varied between individual camels (Fig 3). However, the only individual to have a slope significantly different from the mean was "Camilla" (p=0.0213), the eldest individual in the group and the only female. It should

Fig 1. Camel saddles used by the Bowmanville Zoological Park to provide rides to the public. Two passengers weighing no more than 45 kg combined may ride at once, sitting on either side of the hump. Padding under the seats and on the two straps placed around the proximal and distal torso were used to ensure maximum comfort for the camels.

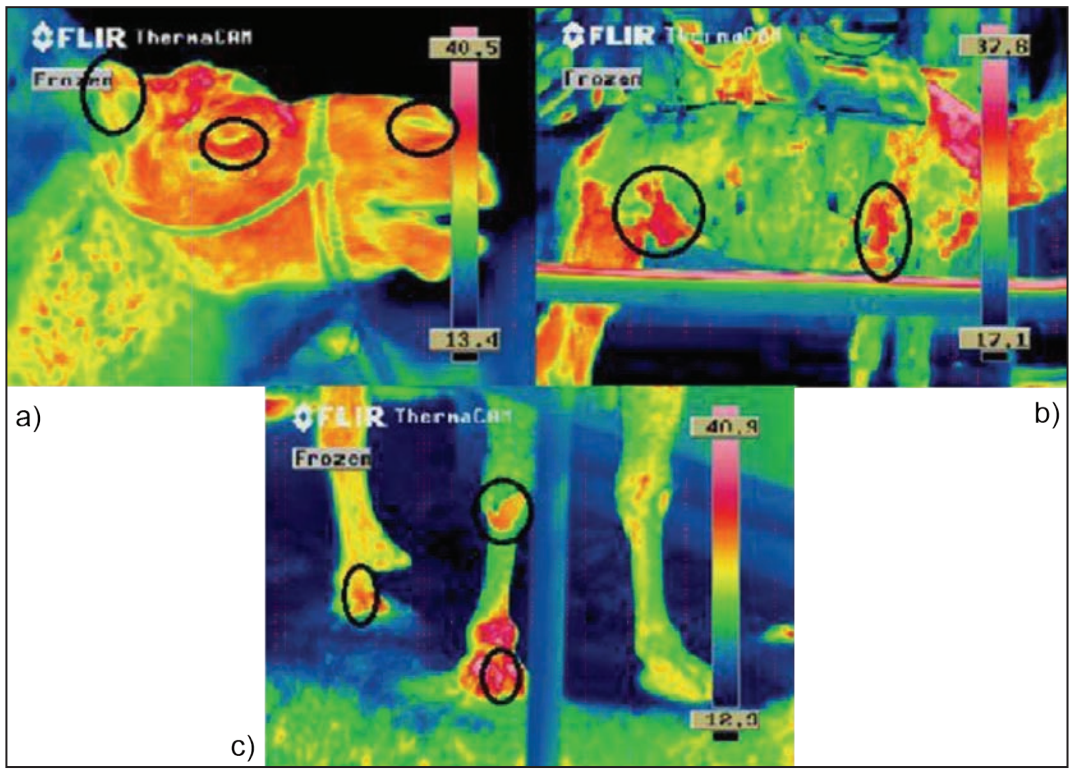

Fig 2. Sample images taken during the current study demonstrating lateral view of the head (a), lateral view of the torso (b) and proximal 45° view of the legs with one complete back foot (c). Areas circled in black show the 8 specific areas where surface temperature readings were measured and recorded.

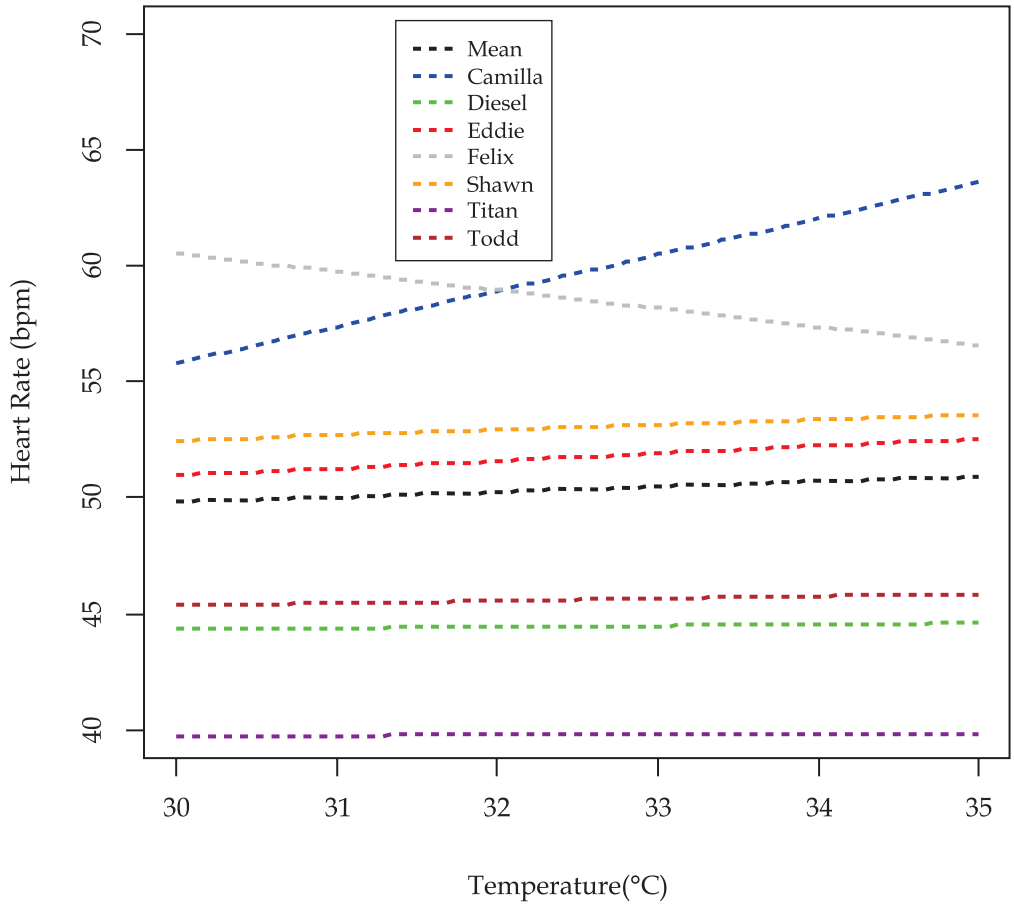

Fig 3. Linear models for each individual camel in the experimental group with heart rate on the x-axis (bpm) and inner thigh temperature on the y-axis (°C). The solid line denotes the linear model mean of the group.

also be noted that the youngest male, "Felix", was the only individual to have a negative slope, although this was not found to be significant (p=0.1496).

Analyses of the thermal images using the ThermCAM software demonstrated a visually large amount of heat loss occurred along the inner thighs and lower abdomen surrounding the urethra (Fig 2). This occurred without regard for the activity level, ambient air temperature, humidity, or presence/absence of direct sunlight on the animal. Overall the inner thigh and lower abdomen were the 4th warmest locations on the body of those examined (following the eye, ear and nostril) as well as being the largest consistent area of heat dissipation based on all thermal images taken.

When a camel was moderately active the average surface temperature was significantly higher overall (1.3%) for all body parts measured (eye 1.3%, ear 2.0%, nostril 1.4%, from foot 3.6%, axilla 5.2% and thigh 1.8%) the exception being the back foot which only showed and average 0.2% increase in temperature (Table 2). Ambient air temperature was associated with the greatest effect on the surface temperature of all body parts (p<0.0001) (Table 3). Humidity appeared to have no effect on the surface temperature of any areas observed with the exception of the thigh (p=0.0150) and the nostril (p=0.0066). The presence of direct sunlight (where shadows are seen) only demonstrated statistical significance when examining the surface temperature of the front foot (p=0.0365) with an average temperature increase of 2.8%. A combination of activity and the presence of sun only demonstrated a statically significance when examining the front foot (p=0.0072) and the axilla (p=0.0010).

Discussion

Montanholi *et al* (2008) was the first to describe a novel use for infrared images that would be useful to the beef production industry. Examination of cattle fount that a prediction of methane production could be made based on heat dissipation, which could then be followed by associating individual feed efficiency with infrared images (Mader *et al*, 2009). These predictions were based on the temperature

of the right and left flank. Based on the results in the present research, there is a significant positive correlation between temperature of the inner thigh and heart rate of camels, suggesting that extrapolation of data from specific areas of thermal images may provide information of the camels' physiological activities, as has previously been shown to be true for cattle (Montanholi *et al,* 2008).

The location of thermal indicator areas differs between cattle and the present observations in camels. While cattle and camels are now both classified in the Order Cetartiodactyla, their overall anatomy and physiology differs (Wilson, 1998). Camels have a localised store of fat on their back, which has significant implications for the vascularisation of tissue and the way in which metabolic heat is dissipated from the body core (Gauthier-Pilters and Dagg, 1981; Wilson, 1984).

Feet have proven to be one of the most important areas in cattle when making predictions from infrared images; however, this is not the case for camels based on the present research. A simple explanation arises by examining the manner in which both species lie on the ground. Cattle, indeed like most other ungulates, rest their lower abdomen on the ground while their back legs rest out from under the body to one side or another (Wilson, 1998). Alternatively, the camel has adapted to lie with legs tucked directly under the body which subsequently raises the abdomen off the ground (Wilson, 1998). This is an adaptation to living in a harsh desert environment in that it allows for a cool passage of air to flow under the abdomen and cool the animals' underbelly (Wilson, 1998). As such, it would make sense to have a large amount of vascularisation in this area in order to dissipate metabolic heat. This is supported by the thermal images (Fig 2) which demonstrate the inner thighs and lower abdomen are the most prominent areas of heat loss for the camel.

The area of the camel's axilla demonstrated the greatest increase in heat loss when ambient air temperature increased. While thermal images did not show the axillary area to be as large an area for heat dissipation as the lower abdomen and inner thighs, it was the most significant based on heat loss per square cm for the regions observed (p=0.0010). The reasons for this may be explained by the specific manner in which the camel rests on the ground (Wilson, 1998). This may have important implications for racing camels that wear saddles. If the saddles'

Table 1. Descriptions of all individual camels used during the study period including ID, sex and age. Individual maximum, minimum and mean heart rate values are shown as beats per minute based on all experimental days and grouped based on activity level.

ID	Sex	Age (Years)	Low Activity Heart Rate (BPM)			Moderate Activity Heart Rate (BPM)		
			Max	Min	Mean	Max	Min	Mean
Diesel	M	16	49	32	38.9	49	42	45.2
Todd	M	8	48	30	39.4	50	39	40.0
Titan	M	7	37	32	34.4	42	32	37.2
Felix+	M	4	48	38	42.7	67	46	57.9
Camilla*	F	19	67	47	53.0	–	–	–
Eddie	M	9	49	36	41.6	–	–	–
Shawn	M	8	49	33	41.8	–	–	–

*Denotes the individual of oldest age and deemed outside of optimal physical peak performance.
+Denotes the individual of youngest age who had not reached physical peak performance.

Table 2. Comparative statistics of camelid body parts analysed in the present study as compared to activity level.

Body Area	Average Low Activity Temperature (°C)	Average Moderate Activity Temperature (°C)	F-value	P-value
Eye	35.0	35.5	9.24	0.0033
Ear	32.3	33.0	4.57	0.0335
Nostril	34.6	35.0	6.78	0.0098
Front Foot	33.6	32.5	7.33	0.0074
Back Foot	33.1	33.0	0.13	0.7151
Axilla	33.4	31.8	14.46	0.0002
Thigh	33.2	33.8	6.75	0.0099

straps overlap onto the axillary region, there could be a substantial decrease in the ability of the camel to properly dissipate heat to the environment and this increase the it's heat load, which in turn could have severe welfare implications for the animal. Care should be taken when designing and placing saddle straps in order to avoid this potential problem. A saddle design, such as that used by BZP for their ride camels, which does not cover the axillary and inner thigh area and where there are two narrow padded straps that go over the ventral thorax and abdomen to hold the saddle in place, should be an ideal saddle design to minimise the impact on thermoregulation in the camel. However, a saddle of any kind is likely to have some impact on thermoregulation. The total effect of this was not measured in the present study and should be examined in more detail before definitive conclusions are reached.

The level of physical activity (LA or MA) performed by the camels in the current study altered the prediction equations in all instances. This is rational, as metabolic activity increases with an increase in physical exertion as has previously been demonstrated (Pond, 1992). Increased physical exertion recruits a higher percentage of metabolically active tissue in response to these demands (Ricklefs *et al*, 1996). As such, future studies should closely monitor or control the levels of physical exertion in order to gain accurate predictions as the sensitivity of this method to activity levels may be high. The physical activity performed by the camels in this study during the ride-giving process, demonstrated an increase in average heart rate of 20%. Heart rate analysis of racing horses has been shown to increase 5-6 times from rest to walk and up to 12 times that of rest during maximal exercise (Butler *et al*, 1993; Physick-Sheard *et al*, 2000). Based on the increase seen in the camels observed for the present study, it is unlikely that the exercise performed here could be classified as greater than moderate; however, more research into the response of camel heart rate to exercise is needed before such a conclusion can be made. It would be of considerable interest in future studies of metabolic activity in camels to include assessment of a higher level of physical activity such as that found in the racing camel, to investigate these predictions.

Humidity only appeared to have a significant effect upon the nostrils (p=0.0066) and the inner thighs (p=0.0150). Camels have an unusual habit of urinating on the inner thighs and back legs during warmer ambient air temperatures, which would act in a similar manner to a kangaroo licking its forearms (Needham *et al*, 1974) in that it would allow for evaporative cooling. An increase in ambient humidity would hinder effective evaporative cooling and instead the warmer urine could increase the surface temperature of the inner thigh (Gebremedhin and Wu, 2001). On examination of the thermal images of camel urination, such an increase on surface temperature due to urination does appear to be the case, however further study is required. Notably, the nostrils, which expel a small amount of water upon exhalation, may have a similar effect as urination and could explain the increases seen in this study (Langman *et al*, 1979). The presence of direct solar radiation (sunshine with shadows) was only a significant factor in the surface temperature of the front feet and only when a camel was moderately active (MA). Throughout the day at the active site, the ride platform provided shade for all areas of the camel except for the front feet, which were almost continuously in the sun during this time. Future studies should perform more frequent measurements of the solar radiation to allow this environmental factor to be taken into account in the data analyses.

Table 3. Descriptive statistics of the linear regression models. Each subsequent regression eliminated the body area which showed the least correlation to the changes in heart rate until only the most significant relationship remained.

Body Area	1st Regression (P value)	2nd Regression (P value)	3rd Regression (P value)	4th Regression (P value)	5th Regression (P value)	6th Regression (P value)
Thigh	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Axilla	0.3373	0.3367	0.3195	0.2874	0.3831	*0.2961
Nostril	0.5199	0.5105	0.503	0.3856	*0.4634	
Front Foot	0.5732	0.5731	0.5613	*0.5401		
Eye	0.6156	0.6157	*0.6137			
Back Foot	0.939	*0.9408				
Ear	*0.9739					

*denotes the least significant value

There was only one female included in this study and it is evident that the regression line estimating the correlation between heart rate and axilla temperature is significantly different from that of the mean model based on males in terms of slope. The female camel was also the oldest camel and has limited mobility and was not involved in giving rides at any time. This female camel was also on long term non-steroidal anti-inflammatory medication (phenylbutazone) to manage her arthritis and this might have affected her results in the study. The youngest male camel included in the current study demonstrated the greatest difference in intercept and slope of all adult male camels, although not significant (p=0.7154). This result may suggest a less reliable prediction measure for individuals who are not within their peak fitness years and for those individuals who have a lower fitness score. Whether, the results seen for the single older female camel in this study was associated with her sex, medication and/or her maturity and lower fitness level is unknown, however, when examined in conjunction with the juvenile male it is suggested that the prediction curves presented here (Fig 3) should be considered in terms of both age and physical fitness. Further research should consider these factors and also examine whether sex makes a significant difference as has been shown in some fish species (Lucas, 1994).

While the use of heart rate telemetry has been used with some success in predicting metabolic activity in other large mammalian species, positive associations are not always the case (Green, 2011; Lucas, 1994; Thorarensen *et al*, 1996). The mammalian heart rate may be susceptible to change based on other environmental factors and particularly stress (Lucas, 1994; Thorarensen *et al*, 1996). The heart rate method used in the present study, has not been reported in any previous camel studies, however, the results presented here suggest a significant increase in individual mean heart rate based on the level of physical activity being performed by the camels. This indicates that the heart rate may potentially serve as an effective indicator of metabolic activity in this species. Future research should aim to perform a calibration study on the camel with consideration for the treadmill method which has been successfully adapted for race horses (Evans *et al*, 1993).

Initial studies with simpler methods would probably be less complicated as well as less detrimental to the welfare of the camels. A calibration curve could offer a better estimate of the prediction equation using the surface temperature values to estimate metabolic activity and some research might not even require a calibration curve (Green, 2011). Additional consideration should be given to substituting heart rate telemetry with triaxial accelerometers, which measure the speed and force with which muscles are used as opposed to secondary oxygen consumption (Eston *et al*, 1998). Accelerometers have demonstrated to be better indicators of metabolic activity, particularly in species with which heart rate telemetry and doubly labeled water methods have failed or are not appropriate. These machines are less expensive than heart rate monitors or water isotopes; however, more validation of this method may be required (Eston *et al*, 1998).

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EXPRESSION PROFILES OF STRESS BIOMARKERS DURING FOETOTOMY IN DROMEDARY CAMELS

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ABSTRACT

Twenty camels subjected to partial foetotomy and 5 camels with spontaneous vaginal delivery (eutocia) were included in this study. All camels with eutocia gave birth to live foetuses. Jugular blood was taken before and after eutocia and foetotomy. Heat shock protein 70 A1 (HSP 70 A1), nitric oxide synthases (NOS), total antioxidant capacity (TAC), glutathione peroxidase (GSH-PX), superoxide dismutase (SOD) and cortisol were determined in the sera using ELISA. Results showed that before foetal expulsion/removal, GSH-PX concentration was higher (P = 0.0001) in the eutocia (185.47 ± 42.5 IU/mL) than in the foetotomy group (13.98 ± 7.1 IU/mL). Similarly, cortisol concentration was greater (P = 0.001) in the eutocia (148.68 ± 2.7 ng/mL) than in the foetotomy group (39.92 ± 12.5 ng/mL). A positive correlation was found between GSH-PX and cortisol concentrations (r = 0.47, P = 0.02). After foetal expulsion/removal, GSH-PX remained higher (P = 0.04) in the eutocia (251.65 ± 112.9 IU/mL) than in the foetotomic (82.06 ± 30.1 IU/mL) cases. Likewise, cortisol concentration was greater (P = 0.00001) in the eutocia (142.30 ± 8.2 ng/mL) than in the foetotomy (30.63 ± 10 ng/mL) group. The other biomarkers did not differ between the 2 groups. Therefore, GSH-PX was the most indicative biomarker for the stress of labour, dystocia and foetotomy in dromedary camels. Expression of cortisol was greater in camels experiencing eutocia than in those suffering dystocia or after foetotomy.

Key words: Cortisol, dystocia, foetotomy, glutathione peroxidase, stress biomarkers

Assisted calving can lead to important metabolic and oxidative changes for the dam and offspring (Nakao and Grunert, 1990; Probo *et al,* 2011; Siddiqui *et al,* 2014; Vannucchi *et al,* 2015). Heat shock protein 70 (HSP 70) was identified as a critical constituent of a very complex and highly maintained cellular defence mechanism to preserve cell survival under hostile environmental situations like infection, inflammation, exercise, exposure of the cell to toxins, starvation and hypoxia (De Maio, 1999; Santoro, 2000; Gulbahar *et al,* 2011; Vinokurov *et al,* 2012; Shen *et al,* 2017). The spontaneous expression of HSP 70 as a vital part of embryo growth has been documented and the existence/lack of HSP 70 influences various aspects of reproduction (Neuer *et al,* 2000; Witkin *et al,* 2017).

Expression of Nitric oxide synthases (NOSs) has been observed in foetal membranes during labour, *in vitro* studies and in patients with preterm pregnancy and twin neonates (Ekerhovd *et al,* 1997; Seyffarth *et al,* 2004; Dugmonits *et al,* 2016).

Glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) are components of the enzymatic antioxidative defense mechanisms against oxidative stress (Agarwa *et al,* 2005; Lubos *et al,* 2011). Oxidative stress has also been reported in cases of toxaemia of pregnancy, retention of placenta, premature birth and after elective Caesarean section (Kankofer, 2001; Georgeson *et al,* 2002; Burlev *et al,* 2014; Gurdogan *et al,* 2014; Nejad *et al,* 2016).

Data concerning the profile of cortisol in humans (Pokoly, 1973; Mastorakos and Ilias, 2003; Nejad *et al,* 2016) and animals (Ghoneim *et al,* 2016) during normal delivery and in dystocia are highly ambiguous and controversial. Moreover, profiles of stress biomarkers like HSP 70 A1, NOS, TAC, SOD, GSH-PX have not yet been documented during foetotomy in dromedary camels. Therefore, the aim of the present study was to explore the expression profiles of HSP 70 A1, NOS, TAC, SOD, GSH-PX and cortisol during foetotomy in dromedary camels.

Materials and Methods

Animals and obstetrical examination

Thirty-six dromedary camels were presented at the Veterinary Teaching Hospital of Qassim University with severe dystocia. The duration of

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labour pain in female camels varied, i.e. within 24 h (n = 2), 48 h (n = 7), 72 h (n = 9) and > 72 h (n = 16). The mean duration of dystocia from onset of labour to admission to the clinic was 98.1 ± 10.4 h. On admission, the dams were examined per vagina for the adequacy of the soft and bony birth ways, while the foetuses were examined for the presentation, position, posture, size and viability. The foetuses were found alive in only three cases (8.3%) and dead in the remaining ones (91.7%). Causes of dystocia were head deviation alone or with double carpal flexion (n = 12, 33.4%), double hock flexion (n = 6, 16.6%), transverse presentation-dorsal (n = 5, 13.9%), narrow cervix (n = 4, 11.1%), uterine torsion (n = 4, 11.1%), double hip flexion (n = 3, 8.3%), double carpal flexion (n = 1, 2.8%) and uterine inertia (n = 1, 2.8%). Foetotomy was decided in 20 of these cases, based on foetal viability, health condition and widening of the soft and bony birth way of the dams.

Foetotomy

Foetotomy was carried out in 20 cases (6 to 15 y; 3 primipara and 17 multipara) using a Thygesen foetotome (Kruuse Embryotome Thygesen, 180011, Longeskov, Denmark). The animals were fixed in sternal position on a special tractor. Foetotomy was carried out using one cut (n = 8), 2 cuts (n = 6) and three cuts (n = 6). Longitudinal cuts were used to remove the deviated head and neck at the base of the neck and the flexed legs below the carpal. Oblique cuts were used for amputating the completely flexed legs at shoulder or hip joints. Foetuses in transverse presentation were first rotated into posterior presentation and then the 2 hind limbs were removed at the hip joints. After partial foetotomy, the rest of the foetus was removed using a calf puller (Stone Ratch-A-Pull, Nasco, Modesto, CA, USA).

Blood sampling and biochemical analysis

Blood samples were taken from the jugular veins 15 min before and 15 min after foetotomy. (Ghoneim *et al,* 2016). In addition, blood samples were taken from 5 female camels with spontaneous vaginal deliveries (8 to 12 y, all multipara). All 5 cases gave birth to live foetuses. The samples were taken from these animals 24 h before and 15 min after foetal expulsion. Serum was harvested and kept at -20 °C until analysis. The HSP 70 A1, NOS, TAC, GSH-PX and SOD were estimated in the camel serum using quantitative sandwich ELISA kits (MyBioSource, San Diego, CA, USA). These kits were specific and validated for camel body fluids. Cortisol was measured in the camel serum using simple solid-phase ELISA kits (Human Gesellschaft fur Biochemica und Diagnostica mbH, Wiesbaden, Germany).

Statistical analysis

The student's t-test was used to compare serum concentrations of HSP 70 A1, NOS, TAC, SOD, GSH-PX and cortisol in the eutocia and foetotomy groups (2-group t-test), before and after eutocia or foetotomy (paired t-test). The data were analysed using the IBM-SPSS program, version 24 (2016).

Results

The mean serum concentration of GSH-PX before foetal expulsion/removal was higher (P = 0.0001) in the cases of eutocia (185.47 ± 42.5 IU/mL) than in those of foetotomy (13.98 ± 7.1 IU/mL). Likewise, the mean cortisol concentration was greater (P = 0.001) in the eutocia (148.68 ± 2.7 ng/mL) than in the foetotomy (39.92 ± 12.5 ng/mL) females. A positive correlation was found between the duration of dystocia and blood cortisol levels (r = 0.35, P = 0.02). Moreover, a positive correlation was also detected between the activity of the GSH-PX and cortisol levels (r = 0.47, P = 0.02) before the time of the intervention. Other estimated parameters did not significantly differ between the groups (Fig 1).

Table 1. Sensitivity, detection range and intra-assay CV% of the kits used for determination of heat shock protein (HSP 70 A1), nitric oxide synthases (NOS), TAC (total antioxidant capacity), SOD (superoxide dismutase), GSH-PX (Glutathione peroxidase) and Cortisol.

Kit	Sensitivity	Detection range	Intra-assay CV%
HSP 70 A1	1 ng/mL	3.12-100 ng/ml	8.7%
NOS	1 U/mL	6.2- 200 U/mL	8.7%
TAC	0.1 U/mL	1.56- 50U/mL	11.5%.
SOD	2 U/mL	15.6-500 U/ml	9.8%
GSH-PX	7.8 U/mL	15.6-1000 U/mL	7.6%
Cortisol	1.5 ng/mL	0.1- 800 ng/mL	6.5%

Females undergoing spontaneous vaginal foetal expulsion (i.e., eutocia) exhibited no significant changes in any of the tested parameters (Fig 2).

After foetotomy, the sole parameter that displayed a significant change in the elevation of its activity was GSH-PX (Fig 3). A positive correlation tended to be found between the number of foetotome cuts and SOD activity (r = 0.34, P = 0.07).

After foetal expulsion/removal, GSH-PX activity remained higher (P = 0.04) in the eutocia (251.65 ± 112.9 IU/mL) than in the foetotomic (82.06 ± 30.1 IU/mL) cases. Similarly, cortisol levels were greater (P =

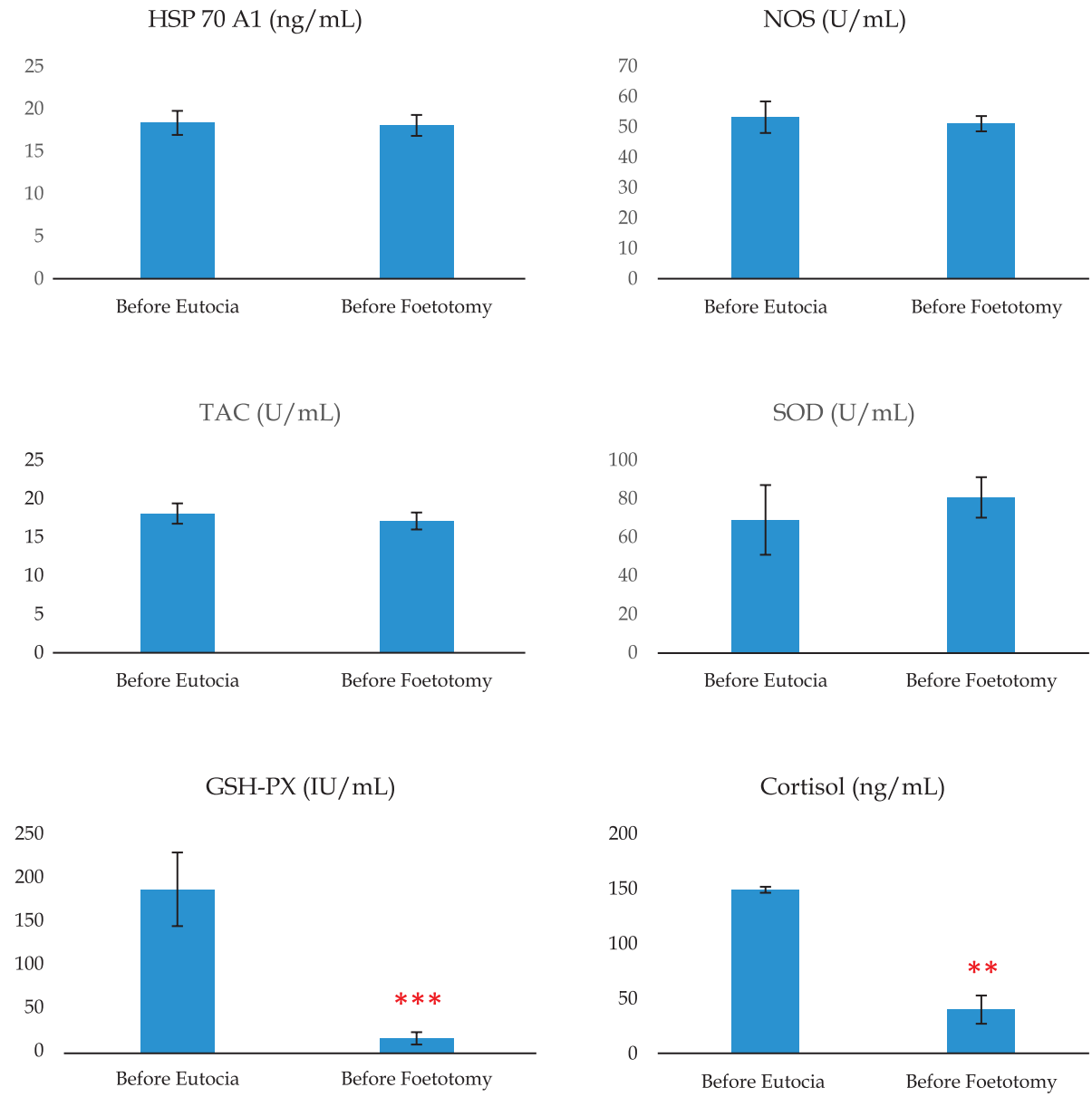

Fig 1. Stress biomarkers (means±SE) in sera of dromedary camels 24h before eutocia (n=5) and 15 min before foetotomy (n=20). HSP 70 A1: heat shock protein, NOS: nitric oxide synthases, TAC: total antioxidant capacity, SOD: superoxide dismutase, GSH-PX: Glutathione peroxidase. ** Significant at P=0.001, *** Significant at P=0.0001.

0.00001) in the eutocia (142.30 ± 8.2 ng/mL) than in the foetotomy (30.63 ± 10 ng/mL) females. A tendency to positive correlation was found between the GSH-PX activity and cortisol concentrations (r = 0.2, *P* = 0.06) following the intervention. Other parameters did not differ between the groups (Fig 4).

Discussion

According to the present data, cortisol levels were higher in eutocia than in dystocia both before and after foetal expulsion/removal. The GSH-PX followed nearly the same pattern as cortisol, with the exception that the GSH-PX activity increased significantly after foetotomy. Indeed, these astonishing findings need careful and particular discussion. Principally, a rise in the cortisol level does not essentially signify that the animals are in a stressful condition (Moberg, 1987); it can also be increased as a result of certain metabolic changes or inflammatory conditions as well as in changes in immune capability (Breazile, 1987). During early and mid-gestation, cortisol mostly arises from maternal origin, while during late gestation it arises mostly from foetal origin. For example, during the first 122 days of gestation in sheep, lamb foetal cortisol is mostly of maternal origin, while by day

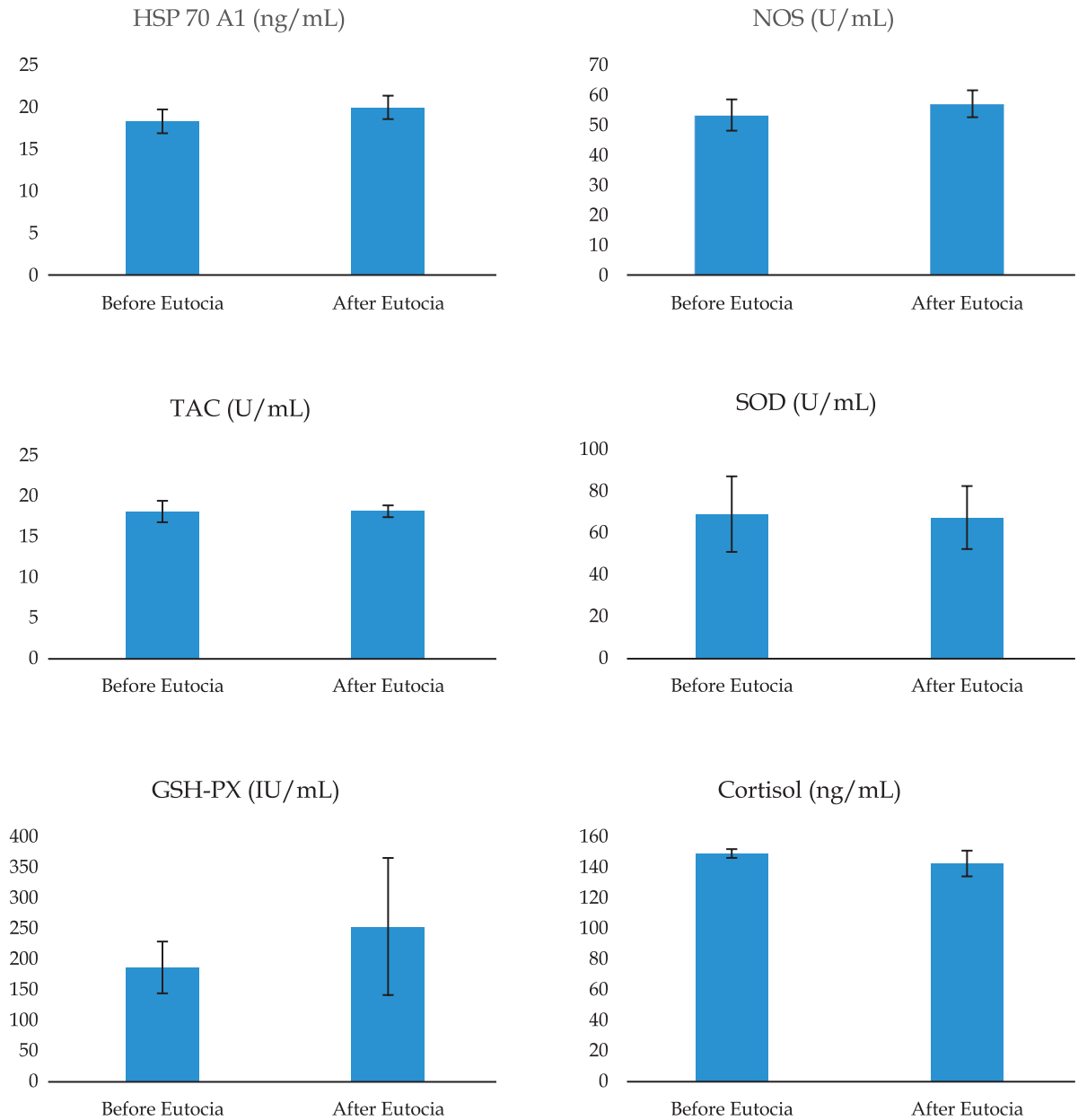

Fig 2. Stress biomarkers (means±SE) in sera of dromedary camels 24h before and 15 min after eutocia (n=5).

136 of gestation, it is mostly (> 88%) of foetal origin (Hennessy *et al,* 1982). On average, the timing of foetal cortisol concentration elevation in sheep is about 12 days before the onset of labour (Magyar *et al,* 1980). It has also been found that high levels of cortisol maximize glucose availability for the foetus and myometrium, which facilitates labour (Benfield *et al,* 2014). As the majority of the camels with dystocia in the current study had dead foetuses, the main source of cortisol in theses dams cannot be attributed to the foetuses; rather, it is most likely ascribed to maternal origin because of the stress of the unresolved dystocia. This might explain why camels with normal vaginal deliveries (with live foetuses, i.e., eutocia females) before foetal expulsion had higher cortisol levels than those with dystocia (i.e., with dead foetuses). This concept was confirmed in women by Goldkrand *et al* (1976). After foetal expulsion/removal, serum cortisol in the dams with eutocia remained high. In humans, maternal plasma corticotropin-releasing hormone (CRH), ACTH and cortisol levels increase during normal labour and drop at about 4 days postpartum (Mastorakos and Ilias, 2003). In goats, a significant rise in cortisol was observed from 12 h before parturition and at parturition, followed by a significant decline one day after delivery (Probo *et al,* 2011). In the present study, foetotomy itself did not alter serum cortisol

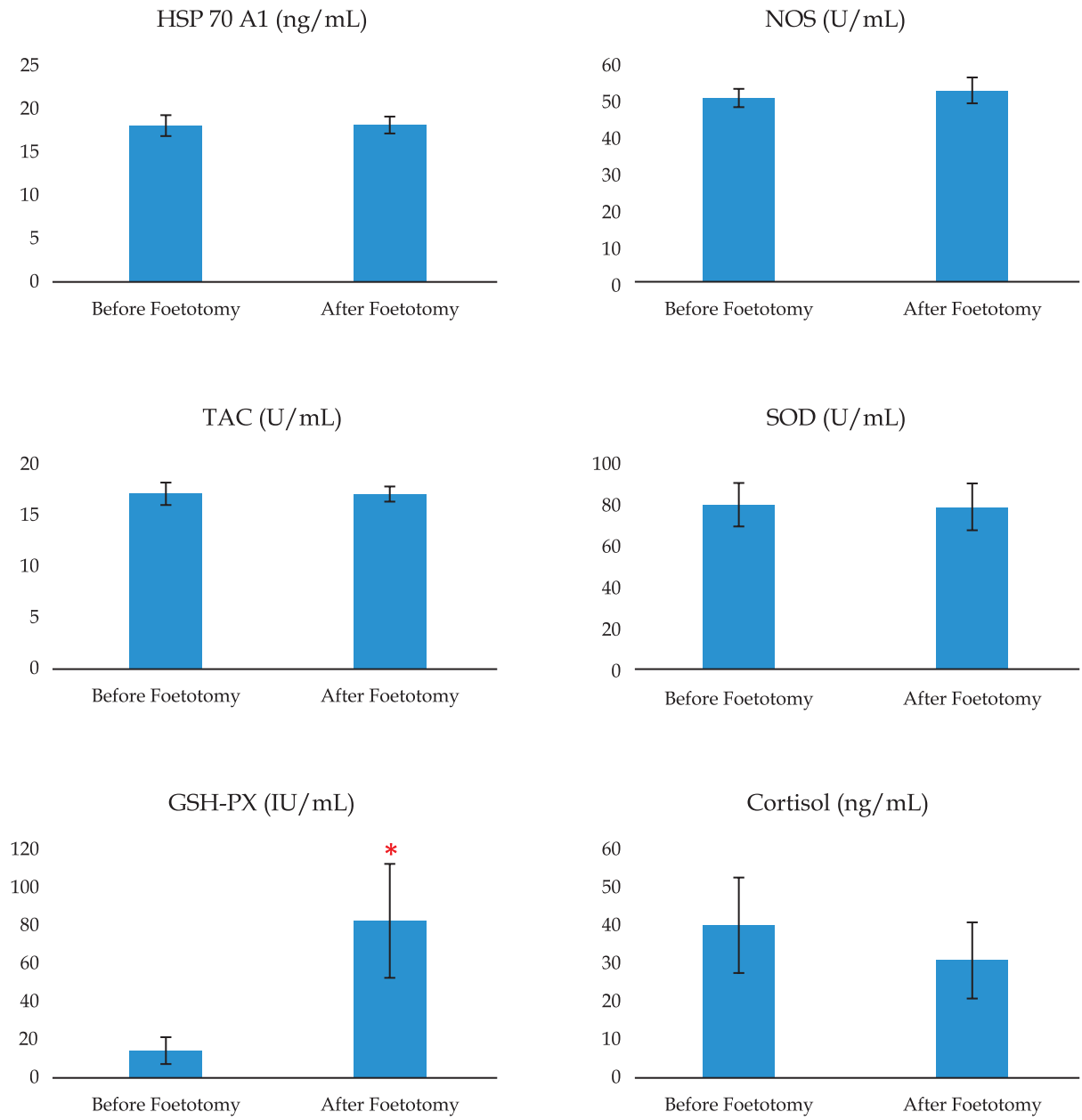

Fig 3. Stress biomarkers (means±SE) in sera of dromedary camels 15 min before and 15 min after foetotomy (n=20). * Significant at P=0.02.

concentration. In light of the above mentioned inducements, the rise in serum cortisol levels in the eutocia dams over those in the foetotomy cases could be explained despite the perception that foetotomy might be more stressful than normal delivery. Hydbring *et al* (1999) observed no differences in the cortisol plasma concentrations between heifers needing assistance and those presenting spontaneous deliveries. These results however, contradict previous reports in cows (Nakao and Grunert, 1990), camels (Ghoneim *et al,* 2016) and goats (Probo *et al,* 2011), where cortisol was higher in animals experiencing dystocia than in those with normal parturition. These authors had suggested that dystocia might be more stressful for animals than would normal parturition. The differences in duration of dystocia in the current study and consequently, the viability of the foetuses might be the causes of these discrepancies.

In the present study, glutathione peroxidase was the most sensitive biomarker for the stress of labour and dystocia/foetotomy. The peroxidase activity of GSH-PX, whose main biological role is to protect the organism against oxidative damage, arises from the reactive oxygen species (Arthur, 2000). It reduces lipid hydroperoxides to their corresponding

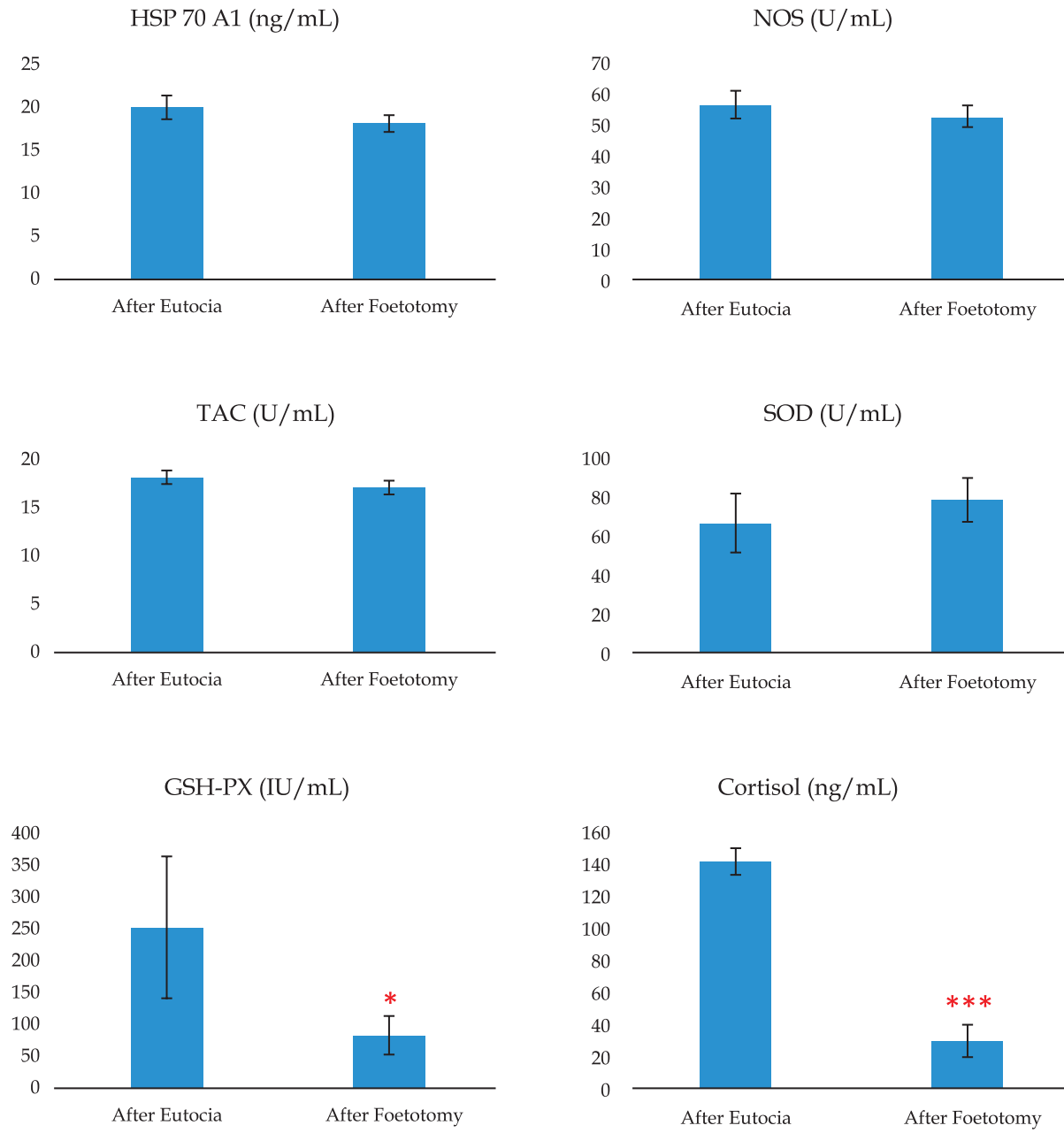

Fig 4. Stress biomarkers (means±SE) in sera of dromedary camels 15 min after Eutocia, (n=5) and 15 min after foetotomy (n=20). *Significant at P=0.04, ***Significant at P=0.00001.

alcohols, thus reducing free hydrogen peroxide to water (Birben *et al,* 2012). The increase of GSH-PX observed in this study might suggest the activation of anti-oxidative mechanisms caused by the imbalance between overproduction and neutralisation of reactive oxygen species. Similar to this result, the GSH-PX showed peak activity around parturition in cattle (Bühler *et al,* 2017) and in women who underwent elective caesarean section and/or their neonates (Siddiqui *et al,* 2014; Nejad *et al,* 2016).

An association between the expression of cortisol and GSH-PX was observed in the current study. This association has also been found during the enzymatic transformation of cortisol with *Bacillus cereus*, where cortisol was transformed to prednisolone and 20,8-hydroxycortisol through the action of 1,2- dehydrogenase and 20-keto reductase systems, respectively (El-Refai *et al,* 1976). Additionally, in one study, coincidental increases in cortisol levels and GSH-PX activity were observed in skiers during the stress of a real competition (Diaz *et al,* 2010).

Although, there have been some reports indicating an association between expression of HSP-

70 A and reproductive failures including chlamydial affection of the fallopian tubes, intrauterine infection, alteration of spermatogenesis, premature ovarian failure, complications of pregnancy, intrauterine growth retardation and preterm birth (Neuer *et al,* 2000; Witkin *et al,* 2017), significant expression of this biomarker was not found either during normal labour in the cases of eutocia or after foetotomy.

Likewise, some previous studies have demonstrated a correlation between the expression of NOS and some reproductive disorders such as preterm pregnancy (Ekerhovd *et al,* 1997; Agarwa *et al,* 2005; Dugmonits *et al,* 2016). However, in this study, the NOS did not change due to the stress of dystocia or foetotomy. The possible explanation of the lack of changes in the other markers in the dystocia cases could be attributed to the variation in the time lapse from the commencement of labour until their admission to the clinic.

In summary, eutocia was characterised by increasing expression of GSH-PX activity and cortisol concentration, which clearly differed from values in the camels with long-standing unresolved dystocia. Foetotomy was associated with a significant rise of GSH-PX activity. Thus, it can be stated that the measurement of the GSH-PX activity in peripheral blood would be a reliable biomarker for monitoring the stress of labour, dystocia and foetotomy in dromedary camels.

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A PRELIMINARY CHARACTERISATION OF A POSSIBLE NON-SURFACE VARIANT SPECIFIC 42kDA ANTIGEN IN *Trypanosoma evansi*

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ABSTRACT

The objective of this study was to investigate the antigenic components of *Trypanosoma evansi* (*T. evansi*) during infection. The antigenic components of intact and trypsin-treated *T. evansi* were identified using a combination of SDS-PAGE and Western immunoblotting against sera from infected rabbits and rabbits immunised with a soluble extract of the parasite. These sera recognised 14 components ranging from ~ 172kDa to ~21.5kDa. A non-trypsin-sensitive component of ~ 42kDa, recognised strongly by sera from infected rabbits and rabbits immunised with the parasite soluble extract was, selected for further studies. This antigen purified by electro elution from acrylamide gels and mono-specific serum produced and used in both Western immunoblotting and enzyme linked immunosorbent assay (ELISA). Serum raised against this antigen recognised only antigenic materials in the homologous *T. evansi* population by both Western immunoblotting and ELISA. Being a non-trypsin-sensitive antigen that was not, cleaved from the parasite by the process of trypsinisation, possibly indicate a non-surface association, yet a variant-specific antigen. The presence of such antigens in *T. evansi* parasites and their role in the process of antigenic variation is discussed. Conclusions were drawn and recommendations were suggested.

Key words: Antigen immunoblotting, Antigen variants, *Trypanosoma evansi*, western immunoblotting

Trypanosomes contain a complex mixture of components many of which recognised as antigens by the host during the course of infection. Some of these antigens are located on the surface of the parasite (Vickerman and Luckins, 1969; Overath *et al,* 1994) or as a part of other morphological structures of the parasites (Muller *et al,* 1992; Yadav *et al,* 2013). The major surface-antigen of the trypanosomes is a variant antigen specific to each particular population (Cross, 1990). The other major trypanosome antigens are invariant and common between stocks of the same species and between different species of trypanosomes. Some of these invariant antigens are located in the surface of the parasite (Radwanska *et al,* 2000; Schwede and Carrington, 2010; El Hassan, 2014), while the majority are non-surface antigens (Sullivan *et al,* 2013). Non-surface antigens studied so far, were, found to be invariant and common between stocks (Laha and Sasmal, 2008) and species of trypanosomes (Eisler *et al,* 1998). The way in which the host deals with the release of internal antigens after VSG-specific lysis is not fully understood, but could form an important part of the response to infection. Once the host has initiated immune-mediated parasite destruction, a range of non-surface parasite components will be, exposed to the host. It is possible that, the type of host response to such individual components might be important in influencing the final outcome of an infection.

To date, a variant-specific antigen, apart from the VSG and to some extent the expression site-associated genes (ESAGs) has not been identified. Antigenic variation of the VSG molecules in Salivarian trypanosomes is the most important mechanism by which the trypanosomes evade the host immune responses. When antibody production against a particular VSG has occurred, trypanosomes bearing this molecule are lysed, but a small proportion of the population switches, expresses a new VSG and eventually become the predominant population in the vascular system of the host (Turner and Barry, 1989).

The objective of this study was to investigate the antigenic components of *Trypanosoma evansi* (*T. evansi*) during infection.

Materials and Methods

Trypanosomes

T. evansi stocks used in this study originally isolated from naturally infected camels in Al-Ahsa

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area, Saudi Arabia. They include one population designated (TR 2257) and 2 variants of another population (TR 2165 and TR 2222). Variants were obtained through several passages of 3 days interval in mice.

Purification of parasites

Trypanosomes expanded in mice were separated from infected blood by anion-exchange chromatography on diethylaminoethyl (DEAE) cellulose column (DE52, Whatman Biochemical, UK) as described by Lanham and Godfrey (1970). The trypanosomes then washed 3 times by centrifugation at 2650g for 20 minutes at 4°C in phosphate saline glucose (PSG, pH 8.0).

Preparation of trypanosome lysate

The parasite crude soluble extract was prepared by re-suspending $2.2x10^9$ DEAE column separated trypanosomes in an equal volume of PSG then freezed to – 80°C for 10 minutes and thawed to room temperature for another 10 minutes. The process repeated 3 times and the lysate was then centrifuged at 10000g for 45 minutes at 4°C. The supernatant removed, its protein concentration determined and aliquoted into 100 µl volumes and stored at -20°C. Detergent lysis (Anderson and Blobel, 1983) using sodium dodecyl sulfate (SDS) sample buffer was used to prepare extracts of whole trypanosome and trypsinised trypanosomes. In this process $2.2x10^9$ DEAE column separated trypanosomes, were diluted with an equal volume of SDS sample buffer, heated to 100°C for 5 minutes, then cooled to room temperature, centrifuged at 10000 g for 5 minutes, aliquoted into 200 µl volumes and stored at -20°C.

Trypsinisation of trypanosomes was performed by adding trypsin (20µl/ml at a concentration of 5mg/ml) to trypanosomes and incubation for 75 minutes at 37°C, under these conditions, the trypanosomes were still intact, motile but their infectivity to mice was completely, abolished, as trypanosomes were not detected by 40 days after inoculation. The trypsin then neutralised by incubation with trypsin inhibitor (20µl/ml at a concentration of 5mg/ml in distilled water) for further 5 minutes at 37°C and the trypanosomes then washed by centrifugation at 5000 g for 5 minutes in PSG.

Electron microscopy of trypanosomes

This was performed following the method of Garnett *et al* (1978). Sixty nm thick sections of trypanosomes embedded in araldite mix: accelerator were cut and mounted on 200 mesh copper grids and stained with uranyl acetate and lead citrate before being viewed and photographed using a Philip 400 transmission electron microscope at 100 kV.

Preparation of T. evansi antisera

Infection serum was, obtained from a rabbit injected intravenously with $1x10^5$ trypanosomes (TR 2165) in 1ml PSG. The rabbit bled for serum prior to infection and on day 2, 5, 9, 14 and 21 post infection. The experiment terminated on day 21 by treating the rabbit with a single intramuscular injection of diaminazine aceturate (BerenilR, Hoechst Lab., Germany) at a dose rate of 7mg/Kg body weight.

Hyper-immune sera to *T. evansi* (TR 2165) crude soluble extract was raised in a rabbit. The immunisation regime was adapted from Harlow and Lane (1988). The rabbit immunised with the crude soluble extract received a subcutaneous injection of 200 µg soluble extract emulsified in Freund's complete adjuvant (FCA) on day one, then boosted by s/c injection with a further 100 µg of the same materials in Freund's incomplete adjuvant (FIA) at 28 and 56 days post-immunisation. A final boost of 100 µg soluble extract in PSG was administered intravenously at 68 days after the first immunisation. The rabbit was, bled for serum on days -1, 7, 10, 21, 34, 39, 49, 61, 67 and 75.

Electrophoresis and Western blotting of T. evansi lysates

T. evansi soluble extract, whole trypanosome extract and trypsinised trypanosomes were subjected separately to SDS-PAGE (Laemmli, 1970) on 7-20% gradient poly-acrylamide gels. Coomassie Blue stain was, used to visualise the protein-banding pattern in each extract. Unstained parasitic components were, electro blotted onto nitrocellulose membranes and detected by Western blotting (Towbin *et al*, 1979) against infection and hyper-immune serum. The serum samples collected from each rabbit pooled equally and then diluted to 1:50 in blocking buffer before used in the assay.

Purification of the 42 kilo Dalton (kDa) antigen

Electro elution from acrylamide gels

Ten SDS-PAGE gels used to provide materials for immunisation. *T. evansi* (TR 2165) soluble extracts separated on 7-20% gradient SDS-PAGE gels stained by Coomassie blue stain and the 42kDa protein located in each gel by reference to a molecular weight standard lane and excised from the gel. The protein then electrophoretically eluted from the excised gel

portion using an electro elution device (Model 422 Electro eluter, Bio Rad. USA) set up according to the manufacturer's instructions. The homogeneity and absence of contaminants in the eluted protein was, assessed by SDS-PAGE using 10% homogeneous acrylamide mini-gel (Mini-ProteinR II, Bio-Rad, USA) prepared according to manufacturer's instructions. Proteins were, visualised by sliver staining. All electro eluates pooled, transferred to dialysis tube (Molecular weight "M.W." cut-off 12000 Daltons, Sigma chemical Co. St. Louis, USA) and dialysed overnight against phosphate-buffered saline (PBS). The total protein concentration of the dialysed protein was measured using a BCA^R test kit (Pierce, USA) and then concentrated ~ 6-fold using a Centrifugal Ultrafiltration System (Sartorius Ltd., Germany) and stored at – 20°C until needed.

High performance liquid chromatography (HPLC)

A $3x10^8$ column separated and washed *T. evansi* (TR 2165) trypanosomes were suspended in 0.015M phosphate buffer, pH 8.0 containing N-CBZ-L-Phenylalanine Chloromethyl Ketone (ZPCK) and n-Octyl β-D-Glucopyranoside (OGP) and centrifuged at 20000 g at 10°C. The supernatant was subjected to fractionation by HPLC. One ml of the detergent solubilised trypanosome materials was injected onto a Spherogel TSK DEAE-5 PW column (Beckman, USA) in 20mM tris (hydroxymethyl) aminomethane (Tris buffer), pH 7.0. Proteins were eluted from the column using a continuous gradient at a flow rate of 1ml/minute over a period of 30 minutes. The absorbance of the column eluate was, monitored continuously at 280 nm and the eluate collected at one minute, intervals. Five fractions representing the major ion-exchange elution peak collected during the first 3-7 minutes were analysed using 8-25% gradient Phast gel (Pharmacia, USA) SDS-PAGE system and the separated proteins were, visualised by sliver staining.

Size exclusion chromatography

The 5 fractions collected from the HPLC ion-exchange column were pooled together and re-fractionated according to molecular size by size exclusion HPLC (Welling and Welling-Wester, 1989) using a Biosep-SEC-S column (Phenomenex, UK). Samples were eluted from the column over a period of 30 minutes using a flow rate of 0.65ml/minute. The absorbance of the column eluate was monitored continuously at 280 nm and the eluate fractions collected at 1 minute, intervals. Nine fractions were collected between 12 and 20 minutes of the chromatography run representing the major elution peak. These fractions were, analysed by SDS-PAGE using a homogenous 15%, acrylamide mini-gel (Mini-ProteinR II, Bio-Rad, USA) prepared and run according to the manufacturer's instructions and proteins were visualised by sliver staining.

Lectin-affinity chromatography

The pool of 5 fractions eluted from the HPLC ion-exchange column was subjected to lectin affinity chromatography using Concanavalin A sepharose 4B (Con A sepharose, Sigma Ltd. UK). Prior to chromatography, 4 ml of the Con A sepharose gel was equilibrated with 10 ml Con A binding buffer pH 6.0. Three millilitres of the sample were mixed with 7 ml of binding buffer and allowed to pass through the column. The column eluate was, passed back through the column a total, of 5 times to wash unbound materials. Material bounds to the column was then eluted by passing 7 ml elution buffer and the eluate was collected as 7x1ml fractions. All fractions were analysed by SDS-PAGE using the Phast system.

Preparation of anti-42kDa antibodies

Two rabbits were, used to produce antibodies to the 42kDa antigen purified by electro elution from stained gels. Stained gels were, used to localise the target antigen band to avoid the risk of contamination by highly immunodominant VSG since the 2 molecules have a large molecular range. Each rabbit received I.M. injection with 100µg antigen emulsified in FCA and this was, repeated 14 days later. The rabbits were, then boosted 3 times by s/c injection with 50µg antigen emulsified in FIA on days 49 and 77 and again with 25 µg of antigen in FIA on day 196. The rabbits were, bled for serum on days -1, 24, 56, 84, 203 and 210.

Monitoring antibody production

An antibody-ELISA was, used to measure the amount of antibody in the sera from the rabbits on days 24, 56, 84 and 203, post primary injection of the antigen. The assay was, also used to determine the titer of the antibodies in the final serum collected on day 210 post-injection. Each serum was tested against a 1/80 dilution of a freeze-thawed soluble extract of the homologous *T. evansi* population as antigen. Sera were, evaluated over a 2-fold dilution range from 1/250-1/8000 for the sera collected 24-203 days and over a 10-fold dilution range from $1/10^2$-$1/10^6$ for the final serum collected at 210 days. In each case, pre-immunisation serum at a similar dilution range was included as a negative control. All serum samples were, tested in duplicates and the antibody

titer of the final serum was, taken as the last dilution that continued to show an OD value of more than two standard deviations (2SD) above the mean of the negative control.

Specificity of anti-42kDa antibodies in day 210 sera

Specificity by antibody-ELISA

Serum collected on day 210 was, tested by antibody-ELISA against freeze-thawed soluble extracts of homologous (TR 2165) and heterologous (TR 2222 and 2257) *T. evansi* populations coated at a dilution of 1/80. Each soluble trypanosome extract was, coated into 30 wells of the ELISA plate. Pre-immunisation serum and PBS/Tween were also included in the plate as negative controls. All sera were, tested at a dilution of 1/1000. The test serum, pre-immunisation serum and PBS were each added to 10 wells of the 3, soluble trypanosome extracts. Mean O.D values of test samples in each trypanosome extract was compared with the mean O.D values of pre-immunisation serum and PBS. Sample with an O.D value of more than two standard deviations (2SD) above the mean of pre-immunisation serum was considered positive.

Specificity by Western blotting

The antiserum was tested by immunoblotting against whole trypanosome extract from homologous (TR 2165) and heterologous (TR 2222 and TR 2257) *T. evansi* populations. The serum samples were diluted to 1/50 in blocking buffer, while the peroxidase-labelled donkey anti-rabbit IgG conjugate was used at a dilution of 1/500.

Specificity by indirect fluorescent antibody test (IFAT)

IFAT (Nadeem *et al,* 2011; OIE, 2012) was used to provide information on variant specificity and location of the 42kDa antigen within *T. evansi* by testing the antisera against homologous (TR 2165) and 2 heterologous (TR 2222 and TR 2257) *T. evansi* populations. Parasitemic whole blood obtained from mice infected with the appropriate *T. evansi* population was fixed either as smears in acetone or in suspension using formalin and was, used as antigen. In both cases, reaction zones were marked on the microscopic slides bearing the fixed trypanosomes. The antiserum was, diluted to 1/50 in PBS before added to the reaction zones. Serum collected from the rabbit infected with *T. evansi* (TR 2165) in section 2.5, pre-immunisation serum and PBS were included on each slide as positive and negative controls. FITC-labelled donkey anti-rabbit IgG at a dilution of 1/40 was, used as a conjugate and the test was performed as described by the above authors.

Specificity by agglutination test

Variant-specificity and agglutinating property of anti-42kDa antigen antibodies was examined by agglutination testing against homologous (TR 2165) and heterologous (TR 2222 and TR 2257) *T. evansi* populations. The serum was tested over a 2-fold dilution range from 1/2 – 1/1024 in a micro-well plate.

Results

Electron microscopy of trypanosomes

Electron microscope sections prepared from untreated trypanosomes were, seen to possess a thick, compact and dense surface coat overlying the plasma membrane. In the sections prepared from trypsinised trypanosomes, the surface coat was, removed leaving behind the plasma membrane, which was sometimes difficult to resolve but the microtubules appeared normal (Fig 1).

Electrophoresis and Western blotting of T. evansi lysates

The protein profile of *T. evansi* lysates resolved by Coomassie blue staining showed a low number of protein bands in the trypsinised trypanosome extract compared to the other 2 extracts (Fig 2, lane 4). Only few proteins including the 42kDa component showed a similar staining intensity to that of the other 2 extracts.

Infection and hyperimmune sera when tested against the 3 trypanosome, extracts using immunoblotting (Fig 3, lanes 1 and 2), recognised 14 components ranging from ~ 172kDa to ~21.5kDa. The majority of the parasite components which acted as antigens during the first 21 days of infection were soluble proteins. Some of these antigens were trypsin-sensitive and were absent from the trypsinised trypanosomes extract. Of the immuno-dominant components of *T. evansi* recognised by the infection serum, the 42kDa component was recognised strongly by this serum in the whole trypanosome extract and to a lesser extent in the other two preparations (Fig 3, lane 1). This component however, was recognised strongly in the 3 preparations by the hyper-immune serum raised against the soluble extract (Fig 3, lane 2).

Electro elution of the 42kDa antigen

The eluate of the gel slices from the 42kDa region in the gel showed a single band of the

appropriate molecular size when subsequently analysed by SDS-PAGE. The amount of protein

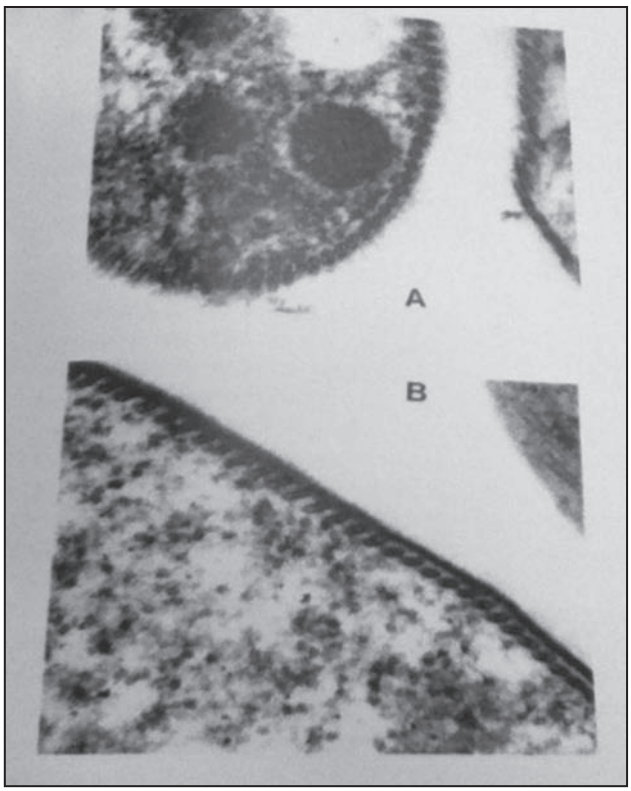

Fig 1. Transmission electron micrograph of section through surface body of *T. evansi* (TR 2165). 1mm ≡10nm.
A-Trypsinised trypanosomes lacking a surface coat.
B-Intact non-treated trypanosomes possessing a surface coat.

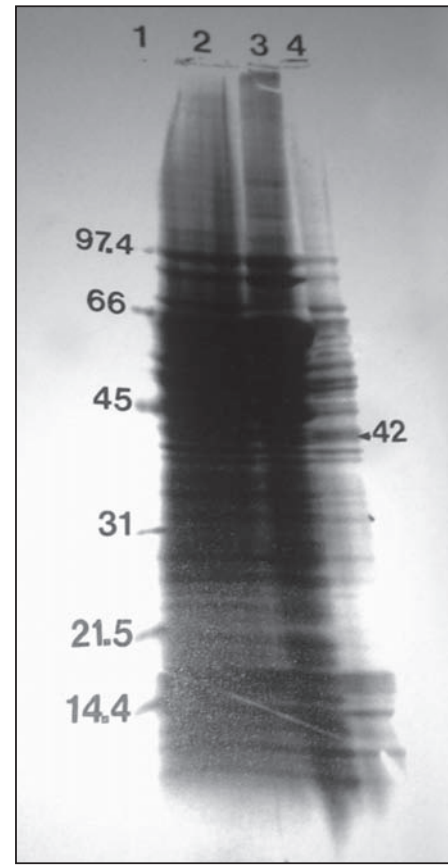

Fig 2. Protein profile of Coomassie stained TR 2165 *T. evansi*
1. Molecular weight marker. 2. Whole trypanosome extract. 3. Soluble extract. 4. Trypsinised trypanosome extract.

obtained following electro elution and ultrafiltration was 671 µg/ml.

Chromatographic purification of the 42kDa antigen

The 5 fractions representing the major elution peak of the HPLC ion-exchange column showed multiple protein bands when analysed by SDS-PAGE Phast gel (Fig 4). Three to nine protein bands per fraction were detected by electrophoresis with molecular weights ranging from approximately 14kDa to 94kDa. The first 4 fractions included the 42kDa target protein (Fig 4, lanes 2-5).

After fractionation of the pooled ionex, fractions 3-7 by size exclusion chromatography 9 fractions were, obtained that constituted the major elution peak. After analysis by SDS-PAGE 1 to 3 protein bands per fraction were identified (Fig 5, lanes 4-10) with a molecular size of approximately 66, 87 and 94kDa.

When the major ion-exchange peak (pooled fractions 3-7) was subjected to lectin affinity chromatography using a Con A sepharose column, the first 6 fractions eluted from the column all contained bands when analysed by SDS-PAGE using Phast system (Fig 6, lanes 2-7). Five protein bands were, identified in each fraction with molecular weights of approximately 15, 30, 62, 66 and 94kDa. The 7th eluate fraction from this column (Fig 6, lane 8) did not contain any protein material resolvable by SDS-PAGE.

Antibody response in the immunised rabbits

Serum from test bleeds collected from the immunised rabbits 10 days following each injection of the antigen showed a progressive increase in absorbance values when tested by ELISA against a soluble extract of the homologous population (Table 1). At a serum dilution of 1/250, almost a 3-fold increase in the absorbance value from day 24 to day 203 post-primary injection was, observed.

The antibody titer of the final serum collected on day 210 post-immunisation was 1/100000 as

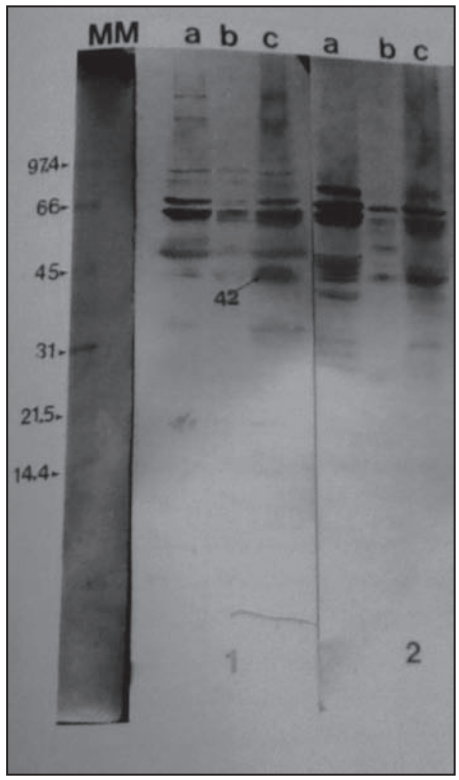

Fig 3. Immuno-dominant components of TR 2165 *T. evansi* **MM.** Molecular weight marker. **1.** Infection serum to intact trypanosomes. **2.** Serum raised against soluble extract. **a.** Soluble extract. **b.** trypsin-treated trypanosomes. **c.** Whole cell extract.

defined by antibody-ELISA against the homologous soluble extract (Table 2).

Characterisation of anti-42kDa antibodies in day 210 sera

Specificity by antibody-ELISA

Serum collected on day 210 from rabbits immunised with the 42kDa antigen when tested for specificity by antibody-ELISA against soluble extracts of the homologous and heterologous *T. evansi* populations reacted only with the homologous population giving an absorbance value of more than 8x that of the pre-immunisation serum (Table 3). In the case of the heterologous populations TR 2222 and TR 2257 absorbance values were less than 2SD above the mean of the negative control (Table 3).

Specificity by Western blotting

Serum collected on day 210 from rabbits immunised with the 42kDa antigen when tested for specificity by western immunoblotting against the whole trypanosome extracts recognised 4 faintly stained protein bands of molecular weight of approximately 65, 35, 29 and 26kDa and a strong

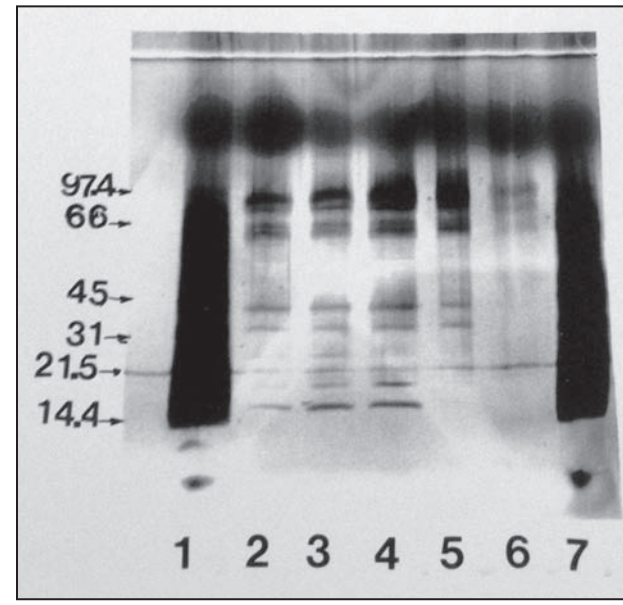

Fig 4. SDS-PAGE analysis of the HPLC ion-exchange chromatography fractions.

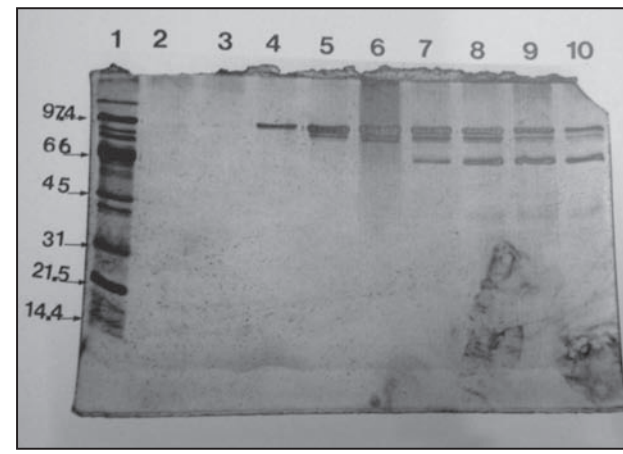

Fig 5. SDS-PAGE analysis of the size exclusion, chromatography fractions.

42kDa band in the homologous TR 2165 population (Fig 7, lane 2). The serum did not recognise any antigenic component in the heterologous populations TR 2222 and TR 2257 (Fig 8, lane 1A and 1B, respectively).

Specificity by indirect fluorescent antibody test (IFAT)

Although, photographs are not shown, fluorescent trypanosomes were detected in both formalin and acetone-fixed preparations of the homologous population (TR 2165) when tested against this serum by IFAT. A strong green fluorescence was observed over the entire outline of both formalin and acetone-fixed trypanosomes. No fluorescent trypanosomes were, detected with the heterologous populations TR 2222 and TR 2257.

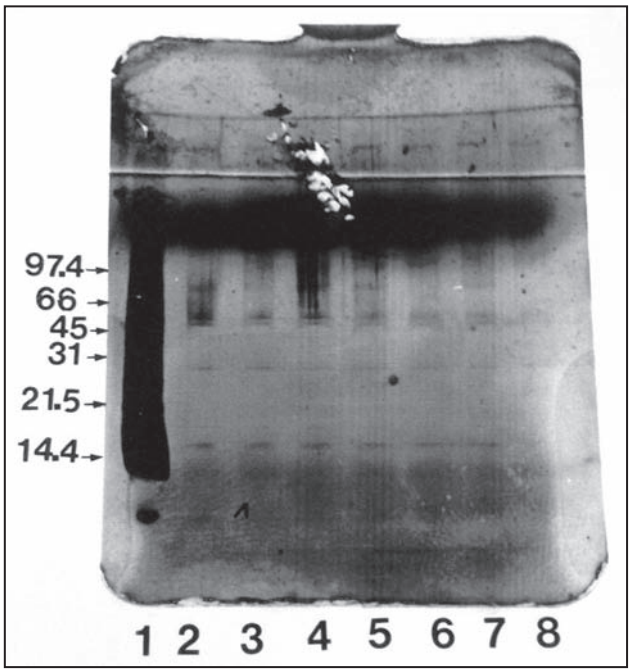

Fig 6. Lectin affinity chromatography fractions analysed by SDS-PAGE

Specificity by agglutination test

The serum did not agglutinate either homologous or heterologous *T. evansi* populations.

Discussion

In prescent study trypsinised trypanosome extract contained the lowest number of *T. evansi* proteins, compared to soluble and whole trypanosome extracts when fractionated by SDS-PAGE and stained with Coomassie stain. Although, trypsin digestion is reputed to cleave mainly the VSG from the parasite surface (Cross, 1975), other proteins were reported to be cleaved during trypsinisation (Frommel *et al,* 1988). Most of the antigens recognised during the course of infection were also immunogens when presented as a soluble extract as they reacted with both infection serum and serum raised against the parasite soluble extract. Differences were however, detected in the antigenicity of the parasite components released during infection and by physical disruption. Some of the parasite components which were not antigenic during the course of infection acted as antigens when presented to the host in soluble extract. This difference in the antigenicity of these parasite materials could be accounted for by difference in the way they were released from the parasite (Barriga, 1981) and the way in which they were presented to the host e.g. immunogenicity of those incorporated with adjuvant will be increased due to their presentation in an aggregated form (Harlow and Lane, 1988).

The 42kDa component of *T. evansi* was not cleaved from the parasite by the process of trypsinisation as was present in the Coomassie stained trypsinised trypanosome extract. This would possibly indicate that it does not possess trypsin sensitive sites, arginine and lysine (Stryer, 1988) and not surface-associated as electron microscope sections prepared in this study from trypsin-treated trypanosomes revealed the removal of the surface coat, leaving behind intact plasma membrane. The 42kDa components was found to be antigenic during the course of infection and immunogenic when administered as a soluble extract as indicated by its reaction with both infection serum and serum raised to the parasite soluble extract. This antigen is also

Table 1. Monitoring antibody response in rabbits immunised with 42kDa antigen tested by Ab-ELISA against homologous *T. evansi* population.

	NRS	Days after primary injection			
		24	56	84	203
Absorbance at 1/250 dilution	0.193 ±0.007	0.773 ±0.002	1.125 ±0.001	1.305 ±0.002	2.028 ±0.001

Table 2. The antibody titre of the final serum collected on day 210 post-immunisation, measured by Ab-ELISA.

Dilution	1/100	1/1000	1/10000	1/100000	1/1000000
Anti-42kDa antibodies	2.36±0.02	1.5±0.02	0.4±0.01	0.1±0.01	0.05±0.002
Pre-immunisation serum	0.2±0.02	0.1±0.01	0.09±0.01	0.02±0.002	0.05±0.002

Table 3. Specificity by antibody-ELISA of serum collected on day 210 from rabbits immunised with the 42kDa antigen.

Trypanosome population	TR2165	TR2222	TR2257
Anti-42kDa antibodies	1.5±0.019	0.181±0.005	0.191±0.02
Pre-immunisation serum	0.181±0.005	0.179±0.004	0.182±0.005
PBS/Tween	0.15±0.005	0.151±0.010	0.153±0.005
2SDEV+mean(Pre-immunisation serum)	0.191	0.187	0.192

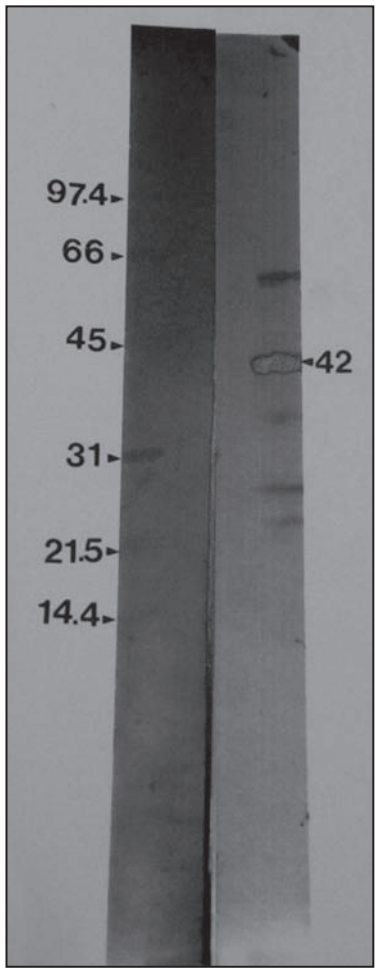

Fig 7. Reactivity of anti-42kDa antibodies tested by Western blotting against the homologous population.
Lane 1: Molecular weight markers. Lane 2: Anti-42kDa antibodies.

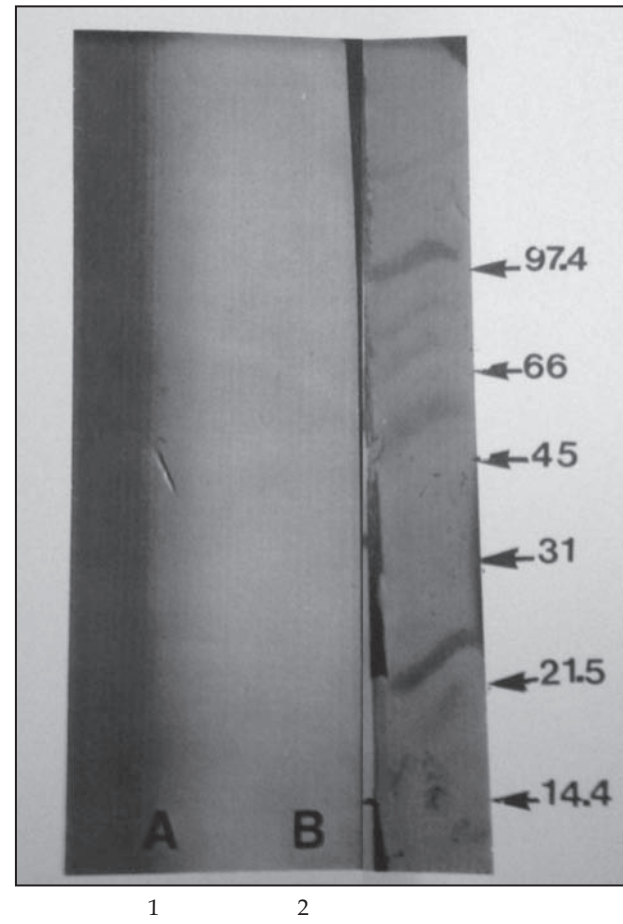

Fig 8. Specificity of anti-42kDa antibodies tested by Western blotting against heterologous *T. evansi* populations.
Lane 1: Anti-42kDa antibodies. Lane 2: Molecular weight markers. A: TR 2222. B: TR 2257.

one of the immuno-dominant antigens of *T. evansi* as indicated by its strong reaction with both types of sera and its presence in the 3 trypanosomal extracts. Immuno-dominant antigens in the range of 41-43kDa were reported in *T. evansi* (Yadav *et al,* 2013).

Immunisation of rabbits with the *T. evansi* 42kDa antigen eluted from Coomassie stained polyacrylamide gels in the present study elicited strong antibody response to the antigen by immunoblotting and ELISA. This result indicates that the antigen retained its immunogenicity and confirms the usefulness of the SDS-PAGE and electro elution for the purification of antigens for immunisation as reported by previous workers (Harlow and Lane, 1988; Kurien and Scofield, 2012).

None of the HPLC chromatographic procedures produced the 42kDa antigen from *T. evansi* lysates in sufficient purity for further study. However, despite the overall failure of this system to separate the antigen, it did provide additional information on the characteristic of this antigen. The isoelectric point (pI) of the target antigen is probably close to 7.0 as it was, eluted during the early stages of ion-exchange chromatography indicating that it was not tightly bound to the column at pH 7.0. The smallest component resolved by size exclusion chromatography was 66kDa. This would be in keeping with the failure to isolate the 42kDa antigen by this method under these conditions. The antigen does not possess any of the sugar residues that are, known to bind Con A such as α-linked D-mannosyl, D-glucosyl and N-acetyl-D-glucosaminoyl (Frommel and Balber, 1987; Chmielewski *et al,* 2014) as it did not bind to Concanavalin A.

The present study has therefore confirmed the usefulness of SDS-PAGE separated proteins as a mean of producing highly specific, high titered antisera to individual trypanosome proteins and highlighted the

problems associated with chromatographic separation of complex mixtures of proteins with similar physico-chemical properties.

The *T. evansi* 42kDa antigen appeared to be a population-specific antigen as serum raised against it only recognised antigenic materials in the homologous population TR 2165 by immunoblotting, ELISA and IFAT. Results from immunoblotting, however, showed that the serum recognised 5 protein bands in the homologous population of the parasite although dominated by the 42kDa protein. Such multiple recognition could be due to contamination of the original gel slice or breakdown products arising from protease activity during preparation of extracts. Contamination from the nearby bands during excision of the antigen from gels is unlikely since the contaminants have a large molecular size range and the target band was excised from a stained gel. The 65kDa contaminant in the present study is clearly not a breakdown product of the 42kDa. Although, the 42kDa component might be a breakdown product of the 65kDa component in the extract used for SDS-PAGE. However, the 42kDa component was present in Coomassie blue stained whole trypanosome extract and trypsinised trypanosome extract that were solubilised by detergent lysis, which is known to hinder protease activity (Sumathi and Dasgupta, 2006) indicating that this component is not a breakdown product of 65kDa. The other components could be breakdown products of either the 65 or 42kDa components.

One of the heterologous populations of *T. evansi* used in the present study was derived from the same stock as the homologous population and known to be antigenically different from homologous population. This suggest that the 42kDa antigen complex is variant-specific. Although, the molecular weight of all but the 65kDa component is lower than the 65-67kDa range reported for *T. evansi* VSG (Uche, 1989). Although, there is evidence that the 42kDa antigen is surface-associated from the overall green fluorescence seen by IFAT, the antiserum did not agglutinate the homologous population of the parasite. This suggest that if surface-associated, the 42kDa is not present in enough sites to cross-link for agglutination formation. It is possible however, that drying and fixation of the trypanosomes during antigen preparation could have exposed the antigen to the serum in IFAT making it appear to be surface-associated.

Results (not presented) from densitometric measurements on Coomassie Blue-stained SDS-PAGE gels indicate that the 42kDa antigen is present in a large amount in the parasite materials and this might also explain the reaction of antiserum in IFAT. Another explanation of the reaction of the anti-42kDa serum in IFAT is that the antigen is possibly sharing an identical epitope with the surface coat antigen. This hypothesis will also explain the importance of the variant-specificity, of the 42kDa antigen. As after switching from one VSG to another the previous VSG will only be gradually diluted during succeeding divisions (Overath *et al,* 1994) and the presence of the 42kDa will possibly lower the chances of VSG-specific antibodies to bind to the surface of the trypanosomes, thereby, allowing the switching process to succeed and the infection to continue.

The *T. evansi* 42kDa antigen reported in the present study may represent, a possible evidence of the presence of non-surface variant specific antigens in this parasite. Further studies, however, are needed to identify the amino acid sequence of this protein and to check whether it corresponds with the VSG sequence. The study also confirmed the usefulness of SDS-PAGE separated proteins as a mean of producing highly specific, high titered antisera to individual trypanosome proteins and highlighted the problems associated with chromatographic separation of complex mixtures of proteins with similar physico-chemical properties.

Acknowledgement

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CHLORTETRACYCLINE IN SERUM AND MILK FOLLOWING SINGLE INTRAUTERINE ADMINISTRATION IN CLINICAL ENDOMETRITIS CAMELS (*Camelus dromedarius*)

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ABSTRACT

This research paper aimed to study the effect of single intrauterine administration of Chlortetracycline (CTC) in clinical endometritis camels (*Camelus dromedarius*) with regard to its levels in serum and milk. CTC pessaries were administered to 5 dromedary camels with clinical endometritis at a dosage of 2 g per animal. Blood and milk samples were collected before treatment and at 12 h intervals for 156 h. Serum and milk were analysed by ultra-performance liquid chromatography (UHPLC/MSMS). The serum and milk analyses revealed that maximum concentration of CTC was detected at 12 h post-treatment. The mean maximum CTC concentration was recorded in the serum and milk at 12 h post CTC administration. The maximum individual concentrations of CTC in milk ranged from 434.0 to 34.6 ng/ml. The mean concentration of CTC in the serum and milk decreased steadily by 24 and 36 h and thereafter post-treatment, respectively. CTC sustained in the milk during the period of 144.4 ± 13.99 h (range, 118 to 154 h) where the serum CTC retained by 111.2 ± 11.70 h (range, 106 to 130 h) after treatment. The mean milk CTC values ≥30 ng/ml was proved till 24 h after treatment (range 12 to 60 h). In conclusion, the safe residual level (≥30 ng/ml milk) established by the US Food and Drug Administration was recorded in the dromedary milk at 24 h onward after intrauterine CTC administration.

Key words: Camel, chlortetracycline, endometritis, milk, uterus

Endometritis is one of the most common uterine disorders of dromedary camels (Tibary *et al,* 2001; Tibary, 2004; Kaufmann, 2005). Numerous antimicrobial compounds have been employed for treatment and prevent of genital infection in the livestock (Pyörälä *et al,* 2014). Attribute to its broad-spectrum antibacterial effect, its efficient action in anaerobic environment of the uterus and activity in the presence of organic debris, tetracycline is recommended for intrauterine treatment (Bretzlaff *et al,* 1983; Bretzlaff, 1987; Hoedemaker, 1998). Although, the absorption and distribution of intra-uterine antibiotic treatment are well documented in cattle (Righter *et al,* 1975; Dinsmore *et al,* 1996; Jaroslav *et al,* 2003), equine (LeBlanc, 2012), and ovine (Cester *et al,* 1996), such studies are rather scarce in dromedary camels. Camels can lactate under severe drought conditions even when dehydrated (Yagil *et al,* 1994). Dromedary daily milk production average is estimated to be between 3 and 10 kg during a lactation period of 12-18 months (Farah *et al,* 2007). Besides the high nutritional quality (El-Agamy *et al,* 1998; Karue, 1998), camel milk is known for its medicinal properties (Magjeed, 2005; Shabo *et al,* 2005; Agrawal *et al,* 2003). The presence of antibiotic residues in milk may have direct toxic effects on consumers (Moats and Medina, 1996; Bencini and Pulina, 1997). The objective of the current study was to determine the concentration of CTC in the serum and milk of dairy camels suffering from clinical endometritis after a single intrauterine therapeutic dose.

Materials and Methods

The present study was conducted at the Camel Research Centre, King Faisal University. Five pluriparous lactating dromedary camels (age 7 to

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12 years and weight 400 to 500 kg) were involved. Camels were maintained under standard conditions of feeding and management. The animals were hand-milked twice a day with 12 h intervals. Their average milk yield was 4.5 litres per animal.

Diagnosis and treatment of endometritis

Camels had a history of failing to conceive after more than 2 services with fertile male camel. All camels were clinically examined by visual appraisal for any signs of abnormal vulval discharge, rectal palpation of the reproductive tract and ovaries (Tibary and Anouassi, 1997), vaginal examination (Tibary and Anouassi, 1997b; Tibary and Anouassi, 2000; Ali *et al,* 2009) as well as transrectal ultrasound (Tibary and Anouassi, 1997a; Tibary and Anouassi, 2000; Tibary *et al,* 2001; Ali *et al,* 2009) using linear-array 5 MHz transducer (UST-588U-5, SSD-500V, ALOKA, Co., Japan). Based on history, rectal, vaginal and ultrasound examination, these animals were diagnosed as suffering from clinical endometritis. Each animal was given a single intrauterine administration of 2g CTC pessaries (MetricyclinKell Belgium).

Milk and blood sampling

Milk samples (25 ml) were collected from the bulk milk of each camel before CTC administration (0 h) and at 12 h intervals up to 156 h after the treatment. Blood samples (10 ml) were taken from the jugular vein of each camel at the same intervals as the milk samples. Serum was prepared by centrifugation at 1400g for 15 min. Milk and serum samples were immediately frozen at – 80 °C and stored for subsequent assay.

CTC analysis

Chlortetracycline was purchased from Sigma–Aldrich, (95%; European Pharmacopoeia HPLC assay, lot 081M1598V, product of China; Shanghai, Trading Co., Ltd.). Mcllvaine buffer was prepared [12.0g sodium hydrogen phosphate (Na_2HPO_4), 3.72g EDTA and 11.9g anhydrous citric acid in 1L deionised water], and the pH was adjusted to 2.9 using concentrated phosphoric acid. Formic acid and methanol (HPLC grade solvent)were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) was obtained from Merck (Darmstadt, Germany). Deionised water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). Disposable 0.22 µm nylon membrane filter (used for extract filtration) were obtained from Millipore (MA, USA).

Extraction and clean up procedures for serum samples was done according to Ghoneim *et al* (2015).

Serum samples (500 µl each) were transferred individually to conical Eppendorf tubes (1.5 ml) and then a volume of 500-µl acetonitrile was added to each tube. The tubes were vortex-mixed for 1 min., and centrifuged at 5000 rpm for 15 min at 4°C. An aliquot of 500 µl of supernatants was transferred to 500 µl mobile phase, mixed and filtered through 0.22µm nylon membrane filter prior to injection onto the UPLC–MS/MS analysis. The injection volume was 7 µl.

Milk samples were collected according to the methods of NaVrátiloVá *et al* (2009) and Cinquina *et al* (2003).

Before the analysis, milk samples were allowed to reach room temperature and stirred for homogenisation then 2ml of each milk sample was mixed with 6ml of McIlvaine buffer. The diluted samples were centrifuged at 4000 rpm and 5°C for 10 minutes to allow complete precipitation of the denatured protein, then after centrifuging; the upper fat layer was removed. The supernatant was decanted from each tube and transferred into a solid-phase extraction (SPE) column (Oasis HLB, Waters, Milford, USA).

SPE Columns were preconditioned (before the addition of samples supernatants) with 3 ml of methanol and 2 ml of water, then an aliquot of supernatants was applied to the column, drained and washed with 1.5 ml 5% methanol in water.

Chlortetracycline was eluted with 2 ml of methanol and then evaporated to dryness on a rotary vacuum evaporator. The evaporated residues were reconstituted in 1 ml of the mobile phase and filtered through 0.2 µm nylon filters for chromatographic analysis. A 7 µl of the filtered extract was injected onto the UPLC–MS/MS system.

UPLC/ESI–MS/MS analysis was done according to Han *et al* (2015). An ultra-performance liquid chromatography (UPLCTM) system Acquity (Waters, Mildford, MA, USA) was interfaced to a triple quadrupole mass spectrometer(UPLC/MSMS) (TQDTM, Waters Micromass, Manchester, UK) using an electrospray interface. The UPLC separation was performed using an Acquity UPLC BEH C18 analytical column, 1.7 µm particle size, 2.1mm × 50 mm (Waters), at a flow rate of 300 µL/min. A gradient elution system was used with mobile phase A (0.1% formic acid in water) and mobile phase B (Acetonitrile) at a total flow rate of 0.3 ml/min. The gradient program was started at 95% mobile phase A and 5 % mobile

phase B, changed linearly to 60 % mobile phase A and 40 % mobile phase B for 3.0 min, changed linearly to 10 % mobile phase A and 90 % mobile phase B for 4.0 min, and finally, 4.1-6.5 min, 5% mobile phase A. The total run time for each sample analysis was 7 min. The injection volume was 7µL. Drying gas as well as nebulising gas was nitrogen. The gas flow was set to 900 L/h. For operation in MS/MS mode, collision gas was Argon 99.995% with a pressure of approximately 2.103 mbar. Capillary voltage of 3.5 kV in positive electro-spray ionisation mode was applied. The interface temperature was set to 450°C and the source temperature to 125°C. Temperature column was set to 40 °C. Dwell times of 30 ms/scan were chosen. Masslynx v 4.1(Waters, Manchester, UK) software was used to process the quantitative data obtained from calibration standards and samples. Quantitation was performed in the multiple reaction monitoring (MRM) mode using peak areas.

MRM transition of chlortetracycline was applied as indicated by Quanpedia software (waters, Mildford, MA, USA) where M/Z 479.4>444.1 and 479.4>462.1 were the qualifier and quantifier ion, respectively.

Specificity:

The specifcity was confrmed based on the presence of the transition ions (quantifer and qualifer) at the correct retention times (that was defined formerly by using one milk sample and one serum sample that spiked at 100ng/ml to check the retention time of chlortetracycline.) corresponding to that of the precursor ion. The measured peak area ratios of qualifer/quantifer were within the range defined in EU Commission Decision 2002/657/EC when compared to the standards.

Matrix-matched calibration curves:

Matrix-matched calibration curves were prepared for control and quantification purposes according to Stolker *et al* (2010) and Han *et al* (2015).

Milk and serum sample extracts (after reconstitution in mobile phase)were spiked with different aliquots of chlortetracycline standard solution to give final concentrations of 6.25, 12.5, 25, 50, 100, 200 and 400 ng/ml. The linearity of the employed method was performed by preparing calibration curves using the aforementioned concentration levels. The calibration curves were constructed by means of plotting the detection response of the matrix matched standard solutions (spiked samples extract) versus the corresponding concentrations by means of regression analysis. From these data, the regression coefficients (r^2) of the calibration curves were calculated where criterion for good linearity should be r^2>0.99.

Method accuracy (recovery percentages):

Method accuracy was determined according to Bousova and Mittendorf (2012), Cinquina *et al* (2003) and Han *et al* (2015) using independently spiked blank samples at three different levels (12.5, 50 and 200ng/ml milk or serum) in 6 replicates. Accuracy was evaluated by comparing found values with standard additions in spikes. Recovery values were expressed in percentages (Table 1).

Table 1. Recovery of CTC in the spiked milk and serum samples (n=6).

Analyte	Spiked level (ng\ml) (milk and serum)	Milk samples (Mean ±SD) (n=6)	Serum samples (Mean ±SD) (n=6)
CTC	12.5	89.40 ± 4.02	89.00 ± 3.09
	50.0	90.50 ± 3.90	94.00 ± 6.05
	200.0	92.30 ± 3.28	91.00 ± 3.57

n= Number of assays, SD= Standard Deviation

Limit of detection (LOD) and limit of quantification (LOQ):

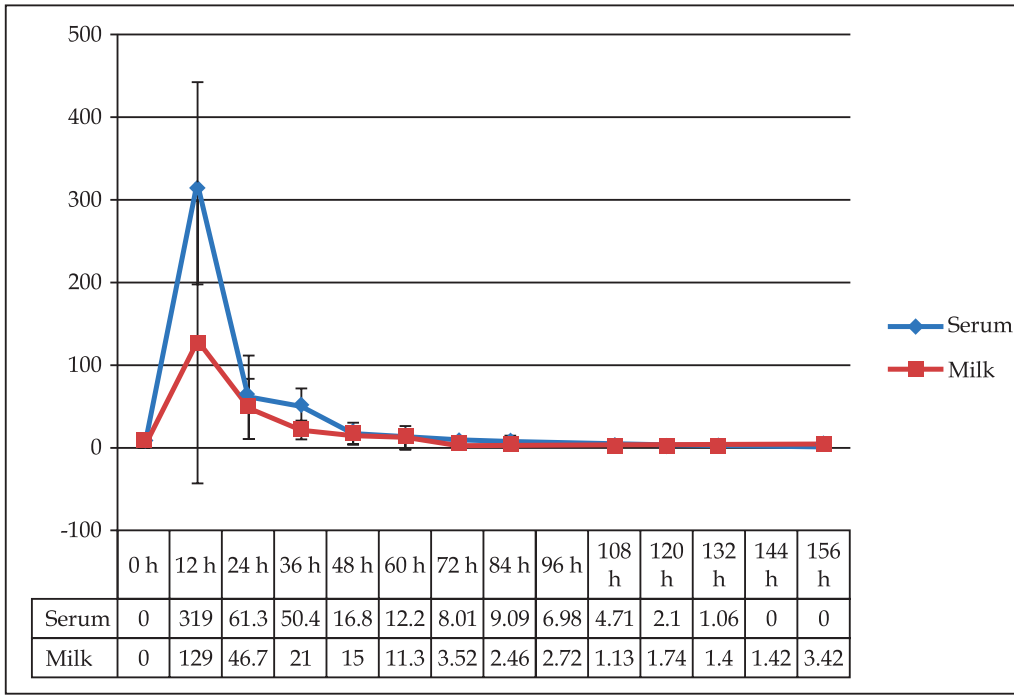
Limits of detection (LOD) and quantification (LOQ) were defined as lowest concentrations with a signal-to-noise (S/N) ratio of ≥3 for LOD or ≥10 for LOQ.

Statistical analysis

Data of CTC are presented as means ± SD. Statistics performed using SPSS program 22.0 statistical software (2013).

Results

The data demonstrated in Fig 1 represented the serum and milk CTC concentrations following intrauterine administration. CTC was firstly detected in the serum and milk 12 h after the treatment. The mean maximum CTC concentration was recorded in the serum and milk by 12 h after CTC administration. The maximum individual concentrations of CTC in milk ranged between 34.6 and 434.0 ng/ml. The mean CTC concentration decreased steadily by 12 h in serum and milk after administration and thereafter. However, after 108 h post-treatment there was no significant decrease in CTC values in milk. CTC sustained in the milk during the period of 144.4 ± 13.99 h (range, 118 to 154 h) where the serum CTC retained by 111 .2 ± 11.70 h (range, 106 to 130 h) after



	0 h	12 h	24 h	36 h	48 h	60 h	72 h	84 h	96 h	108 h	120 h	132 h	144 h	156 h
Serum	0	319	61.3	50.4	16.8	12.2	8.01	9.09	6.98	4.71	2.1	1.06	0	0
Milk	0	129	46.7	21	15	11.3	3.52	2.46	2.72	1.13	1.74	1.4	1.42	3.42

Fig 1. Chlortetracycline concentration in the serum and milk following intrauterine administration in endometritis in dromedary she camels.

treatment. The concentrations of CTC in milk were approximately 70% of maternal serum concentrations. The mean milk CTC values ≥30 ng/ml was proved till 24 h after treatment (range 12 to 60 h).

Discussion

The main principle of practising intrauterine therapy is to achieve a high level of antibiotic in the uterine lumen (Gilbert, 1992; Jaroslav *et al,* 2003). The current experiment was carried out during the non-breeding season to avoid the effect of sex steroids on the blood flow (Dickey, 1997; Bollwein *et al,* 2002) as the rate of drug absorption greatly affected with local blood flow (Morris *et al,* 1993). Recoveries of CTC analyte spiked at 3 different concentrations ranged from 89.4% to 92.3% and 89% to 94.0% in milk and serum, respectively (Table 1). It indicated the extraction and purification method in this study was suitable and reliable for the CTC analyte in milk and serum. The protracted recovery of CTC from the serum and milk (144 and 156 h, respectively) reported in this study was also recorded by Tan *et al* (2007) in cattle. This could be due to slow absorption of the solid form of CTC from the uterus (Roncada *et al,* 2000). On the contrary, tetracycline spray cannot be recovered from the serum of cows after 24 h from intrauterine administration (Girardi *et al,* 1990). The earlier vanishing of CTC from the blood than milk may be attributed to that the concentration of CTC in the serum reflects the concurrent drug absorption from the uterus where its concentration in the milk provides the image over last 12 h. Eliminated tetracyclines in milk were approximately 70% of maternal serum concentrations as reported before (Gideon and Martin, 1997). As the blood concentration of the antibiotic is an indicator for the level in the tissue (Ryan *et al,* 1986; Nix *et al,* 1991), in the current study, the mean serum concentration of CTC recorded in the first 48 h post injection was within the minimum inhibitory concentration required for treatment against bacteria that cause uterine infections in cattle (Sheldon *et al,* 2004). The presence of antibiotics residues in milk poses a dual hazard on the health of consumers (Moats and Medina, 1996; Bencini and Pulina, 1997) as well as dairy products manufacture (Heeschen *et al,* 1991). Activity levels of certain residual antibiotics appear to be decreased by pasteurisation (Roca *et al,* 2010; 2011; Zorraquino *et al,* 2009; 2011). Yet, the effect of pasteurisation on the degradation of CTC is very limited (Loksuwan, 2002; Kellnerová *et al,* 2014). Most camel milk are traditionally consumed as raw milk (Mehaia *et al,* 1995). Although, local and regional authorities and organizations have issued quality requirements (regulations and standards) specific for camel milk (GSO 1970/2009; GSO, 2009). Yet, it does not includ antibiotics residue levels. The safe residual level of ≤ 30 ng/ml in cows' milk established by the US Food and Drug Administration (Popadoyannis

et al, 2000) was recorded in the current study at 24 h onward after CTC injection. On the other hand, the 'safe level' set by the Food and Agricultural Organisation (FAO) and European Union of 100 ng/ml milk (Naoto, 1999) was not reported in the current study. In conclusion, after intrauterine administration of CTC, residues were identified in the milk for 156 ± 13.99 h. The safe residual level of ≤ 30 ng/ml milk established by the US Food and Drug Administration was recorded in milk at 24 h onward after intrauterine CTC administration. Serum CTC retained by 111.2 ± 11.70 h after intrauterine administration.

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CHARACTERISATION OF THE METHANOGENIC ARCHAEAL COMMUNITY IN THE C1 COMPARTMENT OF THE CAMEL (*Camelus dromedarius*)

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ABSTRACT

In depth understanding of the relationship between different methanogenic species in camel rumen is essential as they are most certainly the reason for its low methane emission when compared to other ruminants. The archaeal community inside C1 compartment of camel has not yet been characterised. Here, a *16S rRNA* gene clone library was prepared from the content of the C1 compartment of 6 Indian camels by cloning pooled polymerase chain reaction (PCR)-amplified products. The sequences (n=151) were clustered into 15 OTUs (operational taxonomic units) based on sequencing of unique RFLP pattern and divided into 5 species groups: *Methanobrevibacter* (Mbb.) *millerae* strain SM9, "*Candidatus" Methanoplasma termitum, Mbb. smithii, Mbb. ruminantium, Methanocorpusculum (M.) bavaricum* strain DSM 4179. The genus *Methanobrevibacter* (order Methanobacteriales) was the most prevalent (76.82%), followed by archaea from the orders Methanomassiliicoccales (17.21%) and Methanomicrobiales (5.96%). This study initiates the development of a taxonomic frame of the methanogenic population in the camel. It will inform the manipulation of rumen function to mitigate methane emission while optimising food digestibility.

Key words: *16S rRNA*, archaea, camel, gut, methanogens

The digestive system of camel evolved separately or parallel to ruminant digestion in the suborder Pecora (Bohlken, 1960) having 3 distinct forestomachs (C1, C2 and C3) compared to 4 present in ruminants and also lacking the omasum of true ruminants. Despite, having little resemblance to the rumen, reticulum, omasum and abomasum of true ruminants (Arnautovic, 1997), camel still ruminates and shares some of the anatomical features and digestive physiology with the true ruminants.

Methane is inevitably produced in the course of a normal digestion process facilitated by archaea. The release of methane from the ruminants is the 2nd largest and important anthropogenic source of green house gas in the atmosphere (Moss *et al,* 2000). However, its contribution to global warming remains largely unappreciated. Interestingly, the camel is thought to emit lower quantities of methane than true ruminants (Dittman *et al,* 2014).

While the structure and diversity of archaeal methanogen population has been extensively investigated in livestock ruminants, little is known about the composition of the archaeal community of the camel foregut. Even though they account for most of the earth's biodiversity, majority of microbes, including archaea, are uncultivable, as only ~1% of all microorganisms can be cultured using standard techniques (Hugenholtz *et al,* 1998; Amann *et al,* 1990). Molecular markers, such as *16S rRNA* gene, have been widely used to explore the diversity of environmental methanogens, facilitating the discovery of the complexity of methanogen populations at various sites, also in the gut (Lin *et al,* 1997).

The objective of this study was to characterise the methanogenic archaeal population residing within the C1 compartment of Indian camels using a *16S rRNA* gene clone library.

Materials and Methods

Collection of rumen fluid samples

Approximately 200 ml of the rumen fluid was collected from the C1 compartments of 6 healthy male camels (average age: 4 years; average weight: 372.75±17.70 kg) maintained under intensive system of management at the National Research Centre on Camel Bikaner, India. The animals were randomly selected from a group of 328 camels and had no recorded history of disease. Samples were collected in duplicate after 4 h of feeding on 4 kg of concentrate guar husk (*Cyamopsis tetragonoloba*) and groundnut

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straw (*Arachis hypogea*) in 1:1 ratio. The water was given *ad libitum*. The samples were collected after the approval of the institutional ethical committee. The animals were tranquilised by intravenous injection of xylazine (5 ml of 20 mg/ml solution per animal). The rumen fluid was sampled using a rumen fluid extraction unit especially designed for ruminants (A. J. Enterprises, Chennai, India). The samples were immediately transferred to the laboratory, on ice. These were then strained through a doubly folded muslin cloth to remove the debris and were subjected to further fractionation following the procedure of Martin *et al* (1994).

Isolation and amplification of DNA

DNA was isolated from the samples using a QIAamp® DNA Stool mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out in 0.2 ml thin-walled microcentrifuge tubes, in a Mastercycler Gradient 5331 (Eppendorf, Hamburg, Germany). Methanogen-specific primers Met86F (5'-GCTCAGTAACACGTGG-3') and Met1340R (5'-CGGTGTGTGCAAGGAG-3') (Wright and Pimm, 2003) were used for the amplification of the *16S rRNA* gene, as described earlier (St-Pierre and Wright, 2012).

Cloning, screening and RFLP analysis of amplified methanogen sequences

The amplified 1.2-kb products were purified using NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL, Düren, Germany) and pooled. These were then cloned into pGEM®-T Easy Vector (Promega, Madison, WI, USA) and used to transform *Escherichia coli* JM109 competent cells (Promega), according to the manufacturer's instructions. White colonies (n=163) were selected by blue-white screening of ~200 colonies. Plasmids were isolated from all the white colonies using XcelGen-Plasmid mini kit (Xcelris Genomics, Ahmadabad, India) and the integrity of isolated plasmids was analysed by 1% agarose gel electrophoresis. No plasmids were obtained from 12 colonies. Colony PCR using gene-specific primers (Met86F and Met1340R) and conditions mentioned above was then performed. The PCR products were digested with 2.5 U (0.25 µL) of *Hae* III restriction enzyme (Thermo Scientific, Waltham, MA, USA), overnight at 37 °C (Wright and Pimm, 2003). The digested products were electrophoresced on 2% agarose gel and grouped according to their restriction profiles. PCR products representing 20 representative restriction fragment length polymorphism (RFLP) profiles were then sequenced.

Sequencing and phylogenetic analysis of methanogen sequences

Fifteen representative PCR products with unique RFLP profiles were used as templates in Sanger sequencing. BigDye terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) was used for the sequencing reaction. Samples were analysed on ABI 3730 xl DNA analyser at Xcelris Genomics. ChromasLite version 2.1.1 (Technelysium Pty. Ltd., South Brisbane, QLD, Australia) was used to analyse *16S rRNA* gene sequences of the methanogens. The DNA sequences were assembled into contigs of ~1200 bp. Low-quality base calls from the beginning and end of each sequence were removed by CodonCode aligner V.5.1.5 (Centerville, MA, USA). Sequence similarity searches were carried out using the BLAST alignment search tool from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi; last accessed on 10 August 2016). Each unique clone was designated as "NRCCMET", followed by the clone number, in the order in which these were sequenced.

Accession numbers

All novel sequences obtained in the course of the study (n=15) were submitted to the NCBI database. They may be found under the GenBank accession numbers KT164812–KT164826.

Results and Discussion

In this study, methanogenic diversity within the C1 stomach compartment of the camel was investigated by constructing partial sequence clone library of the *16S rRNA* gene. A total of 15 OTUs were identified by PCR-RFLP and sequencing (Fig 1, Table 1).

Comparative analysis of the camel methanogen and reference sequences

In majority, 76.82% (i.e.116 clones) of the cloned unique 151 sequences shared similarity with *Methanobrevibacter* spp. (Table 1). Based on a comparative analysis, the sequences were divided into 15 Operational Taxonomic Units (OTUs). The largest group comprised 8 OTUs (1, 3, 4, 8, 9, 11, 13 and 15; 86 clones; 56.95% of all clones) that shared ≥97% similarity with *Mbb. millerae* strain SM9 (Fig. 2). Three OTUs (2, 5 and 10; 26 clones; 17.21% of all clones) were found to be 93% identical with *"Candidatus" Methanoplasma termitum*. Two OTUs

(6 and 7; 22 clones; 14.56% of all clones) were 93% identical with *Mbb. smithii*. One OTU (12; 8 clones; 5.29% of all clones) shared ≥97% similarity with *Mbb. ruminantium*. Finally, OTU 14 (9 clones; 5.96% of all clones) showed 97% similarity to *M. bavaricum* strain DSM 4179 (Table 1).

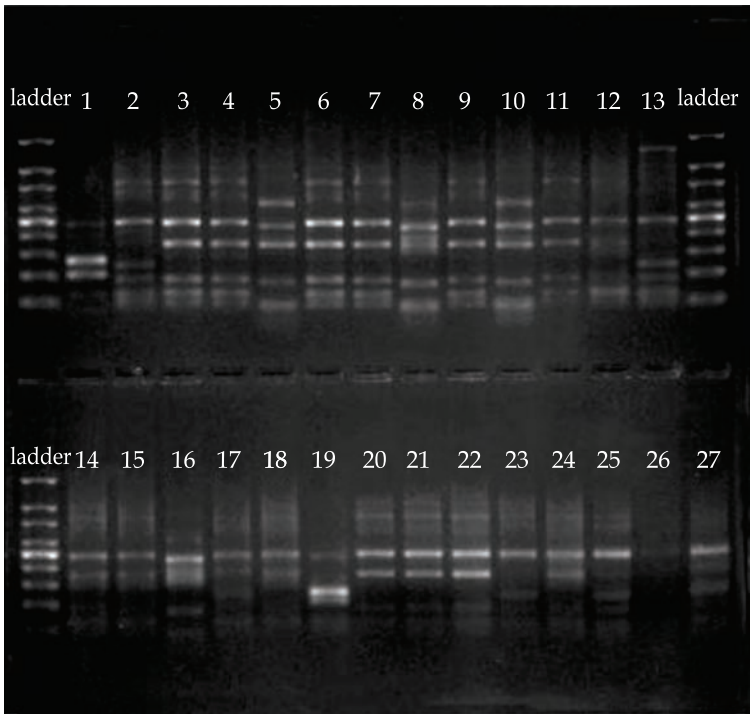

Fig 1. Restriction fragment length polymorphism (RFLP) analysis of cloned 16 rRNA genes. HaeIII-digested PCR products were resolved on 2% agarose gel. Ladder, 100 bp ladder; lanes 1–27, a subset of different RFLP profiles (from 151 samples analysed).

Our findings suggest that the reported species comprise the normal ruminal flora of the camel. Further, the uncovered methanogenic diversity indicates that use of the universal primers for the construction of clone libraries was justified, as the investigation was not prejudiced against a particular species. To the best of our knowledge, this is the first report characterising the methanogenic archaeal diversity in the C1 compartment of the camel employing a *16S rRNA* gene clone library.

The diversity of the cloned camel sequences

Microbes from the genus *Methanobrevibacter* are the predominant ruminal methanogens worldwide and have been detected in dairy cows (Tatsuoka *et al,* 2004; Jeyanathan *et al,* 2011; King *et al,* 2011; Ozutsumi *et al,* 2012; Seedorf *et al,* 2015), Mediterranean water buffaloes (Franzolin *et al,* 2012), sheep (Seedorf *et al,* 2015; Wright *et al,* 2004; Wright *et al,* 2008; Yanagita *et al,* 2000; Snelling *et al,* 2014), yak (An *et al,* 2005), reindeer (Sundset *et al,* 2009a) and marsupials (Evans *et al,* 2009). Indeed, in this study, 56.95% of all clones shared ≥97% similarity with *Mbb. millerae* strain SM9. This suggested that *Mbb. millerae* is one of the most prevalent species in the C1 compartment of the camel. Similarly, the dominance of OTUs sharing >98% identity with *Mbb. millerae*

Table 1. The nearest sequence identity of *16S rRNA* gene library clones from the camel foregut.

16S rRNA gene OTU	Total clones	GenBank accession no.	Nearest valid taxon	%Sequence identity
NRCCMET1	7	KT164812	*Mbb. millerae* strain SM9	98
NRCCMET2	8	KT164813	*"Candidatus" methanoplasma termitum*	93
NRCCMET3	23	KT164814	*Mbb. millerae* strain SM9	97
NRCCMET4	9	KT164815	*Mbb. millerae* strain SM9	98
NRCCMET5	9	KT164816	*"Candidatus" methanoplasma termitum*	93
NRCCMET6	9	KT164817	*Mbb. smithii* TS96A	93
NRCCMET7	13	KT164818	*Mbb. smithii* TS96A	93
NRCCMET8	10	KT164819	*Mbb. millerae* strain SM9	99
NRCCMET9	9	KT164820	*Mbb. millerae* strain SM9	98
NRCCMET10	9	KT164821	*"Candidatus" methanoplasma termitum*	93
NRCCMET11	9	KT164822	*Mbb. millerae* strain SM9	99
NRCCMET12	8	KT164823	*Mbb. ruminantium* M1	97
NRCCMET13	9	KT164824	*Mbb. millerae* strain SM9	99
NRCCMET14	9	KT164825	*Methanocorpusculum bavaricum* strain DSM 4179	97
NRCCMET15	10	KT164826	*Mbb. millerae* strain SM9	98

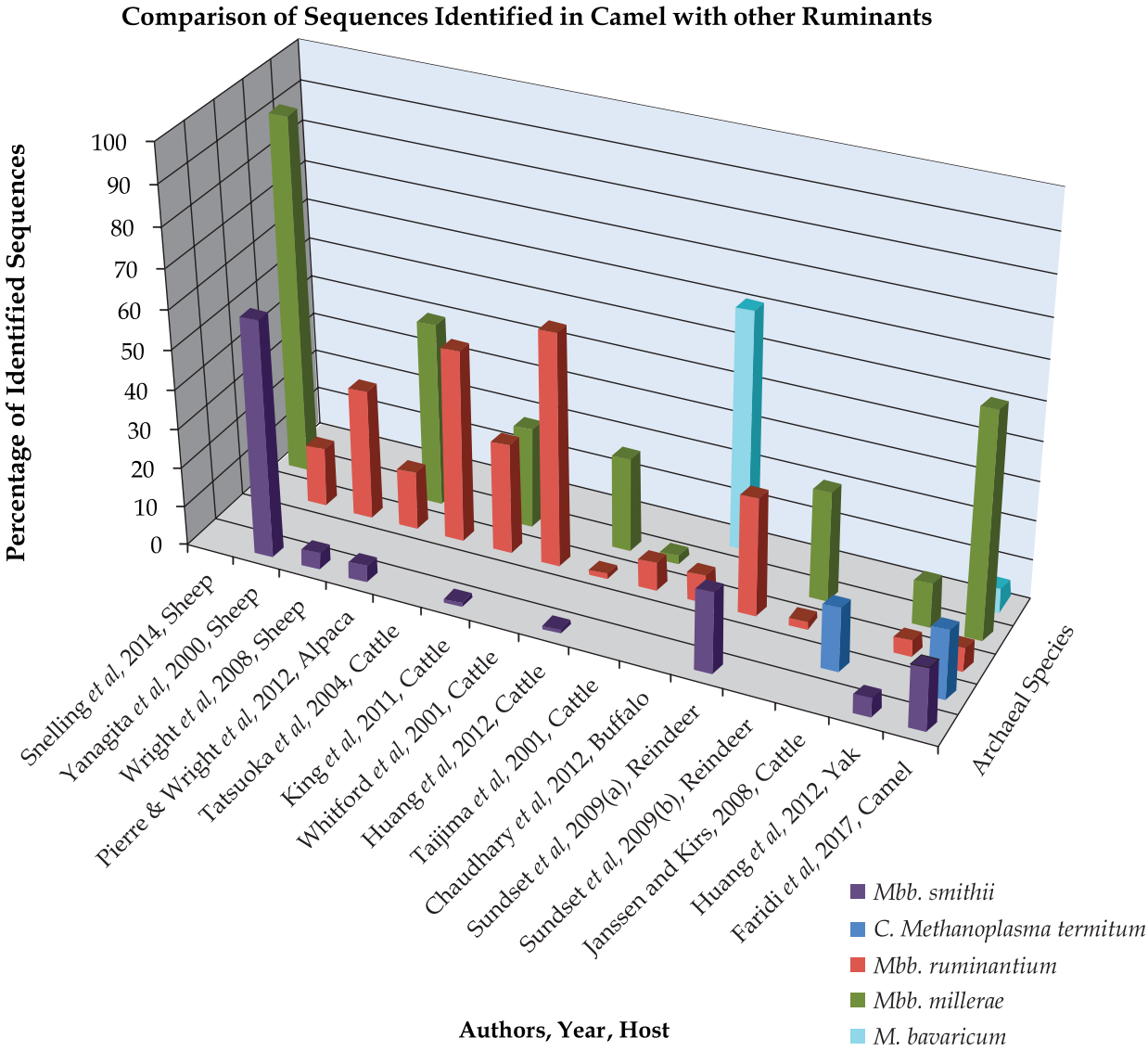

Fig 2. Comparison of identified sequences in camel with other ruminants.

was reported in the foregut of the North American alpaca (St-Pierre and Wright, 2012) (Fig 2) Although, alpacas and dromedaries are present in different geographical locations, yet the predominance of *Mbb. millerae* clones in both animals suggested that the ruminal methanogenic population has little to do with geographical location and common ancestry and unique digestive anatomy might be the reason for dominance of *Methanobrevibacter* spp. in both animals.

In a study of yak and cattle from Qinghai (Tibetan plateau, China), 48% sequences from the order Methanobacteriales, shared 97–98.3% sequence identity with *Mbb. millerae*, whereas in cattle, nearly 82% of the sequences shared 96- 99% identity with *Mbb. millerae* (Huang *et al,* 2012). Similarly, in Svalbard reindeer grazing on natural pastures, 28% of the clones shared 97–98% sequence identity with *Mbb. millerae* (Sundset *et al,* 2009b). *Mbb. millerae* strain SM9 was also identified in Australian sheep fed 3 different diets (Wright *et al,* 2004). *Mbb. gottschalkii* clones were identified in the cattle and reindeer and *Mbb. thaueri* and *Mps. stadtmanae* clones were identified in the reindeer; however, these were not detected in the camel. Geographical effects might explain such host-specific species association.

In the persent study, 8 clones comprised 1 OTU shared 97% similarity with *Mbb. ruminantium*. This is one of the most-widely identified species of the order Methanobacteriales. It is found, in pasture-grazing sheep in Western Australia (St-Pierre and Wright, 2012), in feedlot cattle in Canada, fed different diets (corn-fed Hereford-cross cattle from Ontario and potato-fed Hereford-cross cattle from Prince Edward

Island) (Wright *et al,* 2007) and in purebred sheep in Venezuela (Wright *et al,* 2008).

Methanobrevibacter was also the dominant genus in the cattle rumen in Sweden although, sequences related to *Mps. stadtmanae* were also present (Danielsson *et al,* 2012). Zhou *et al* (2009; 2010; 2011) reported *Methanobrevibacter* to be the most abundant genus in the cattle rumen, along with *Methanosphaera*. No clones sharing similarity with *Methanosphaera* spp. were found in the camel of persent study.

On the Indian subcontinent, Methanobacteriales and Methanomicrobiales were reported to be the most dominant orders of methanogens in Indian crossbred cattle and buffaloes (Chaudhary *et al,* 2012a). In one study concerning Murrah buffaloes in northern India, 15 OTUs comprising 94% of total sequences were >98% identical to *M. mobile* and rest 2 phylotypes showed similarity to *Thermoplasma acidophilum* and *M. bavaricum*, which taxonomically also falls under the order Methanomicrobiales but belongs to the family Methanocorpusculaceae (Chaudhary and Sirohi, 2009). From the 151 clones obtained from camel rumen, one OTU (nine clones) was found to share 97% sequence similarity with *M. bavaricum* strain DSM 4179. Similarly, based on ARDRA (Amplified Ribosomal DNA Restriction Analysis) patterns, *M. bavaricum* was reported to be the most abundant species in the rumen of northern Indian Murrah buffaloes maintained on a standard diet but having less than 97% sequence similarity. The 2nd largest phylotype contained 24 clones, 92–99% identical to *M. mobile* (Chaudhary *et al,* 2012b). No phylotype resembling *M. mobile* was detected in the camel in this study. One OTU comprising 6.4% of the total clones were found related to *Mbb. ruminantium* in that study, while 2 OTUs comprising 5.29% of the total clones were related to *Mbb. ruminantium* in this study. This apparent shift of abundance from genus *Methanomicrobium* to *Methanobrevibacter* in camels can be attributed to their different digestive anatomy from true ruminants and high feed efficiency on low resources.

Later studies of 3 Surti buffaloes from Gujarat (India) also identified clones belonging to Methanomicrobiales and Methanobacteriales (Singh *et al,* 2011; Singh *et al,* 2012). Similarly, Singh *et al* (2013) reported that *Methanomicrobium* was the most abundant genus in Surti buffaloes. Nearly 62% sequences, belonged to the *Methanomicrobium* genus; 14.28% sequences belonged to the *Methanobacterium* genus and nearly 24% of the sequences belonged to uncultured methanogens. This is in contrast with our study, where only 5.96% of the total sequences were related to the order Methanomicrobiales (family Methanocorpusculaceae) and most of the sequences belonged to the genus *Methanobrevibacter*. Nevertheless, Methanobacteriales was shown to be the dominant order in Surti buffaloes (Singh *et al,* 2010) and Karan Fries cattle (Sirohi *et al,* 2013); and also most predominantly identified order in the camel as well.

In camel, 3 OTUs were 93% related to *"Candidatus" Methanoplasma termitum*, an obligate hydrogen-dependent methylotroph from the newly discovered 7th order of methanogens, consisting of a novel uncultured lineage, Methanomassiliicoccales (previously known as Methanoplasmatales, TALC, or Rumen Cluster C, RCC). Uncultured thermoplasmatales were identified in the cattle rumen (Tajima *et al*, 2001; Wright *et al,* 2007; Janssen and Kirs 2008); isolate ISO4-G1, 80% related to *"Candidatus" Methanoplasma termitum*, was isolated from the sheep and cow rumen (Jeyanathan *et al*, 2011); and sequences sharing 81.3% similarity with *Thermoplasmata* were found in the termite hindgut (Shinzato *et al*, 1999). However, the presence of this species in the C1 compartment of the camel has not been reported until now.

The phylogenetic analysis of *16S rRNA* gene library revealed that the order Methanobacteriales is the most abundant phylotype in the camel's gastrointestinal tract under normal dietary conditions. The 2nd most prevalent group comprises *"Candidatus" Methanoplasma termitum*. Many studies from India have reported that archaea from the order Methanomicrobiales are the most predominant methanogens in the cattle and buffaloes, but this was not observed in this study.

The significance of exploring archaeal diversity within the camel foregut lies in linking the presence of these archaeal communities to enteric methane emission and digestive efficiency. Based on our data, it would be tempting to speculate on the methanogenic potential of the identified species. Judging by their reported substrate utilisation, these species might be classed as hydrogenotrophic methanogens, i.e., using hydrogen or formate (*Mbb. millerae*, *Mbb. smithii*, *Mbb. ruminantium*, *M. bavaricum*) and methylotrophic methanogens, i.e., using methanol or other methyl compounds (*"Candidatus" Methanoplasma termitum*) (Boone *et al*, 1993a).

In this study, all experimental animals were fed the same diet, with no seasonal variation.

Experiments on feed digestibility and characterisation of methanogens in other camel flocks are required to elucidate the relationship between these microbes and methane emission in the camel. The scope for future research also includes the identification of genes encoding enzymes involved in methanogenesis, e.g., methyl-coenzyme M reductase and formylmethanofuran transferase, to facilitate the understanding of mechanisms that mediate digestion in the ruminants.

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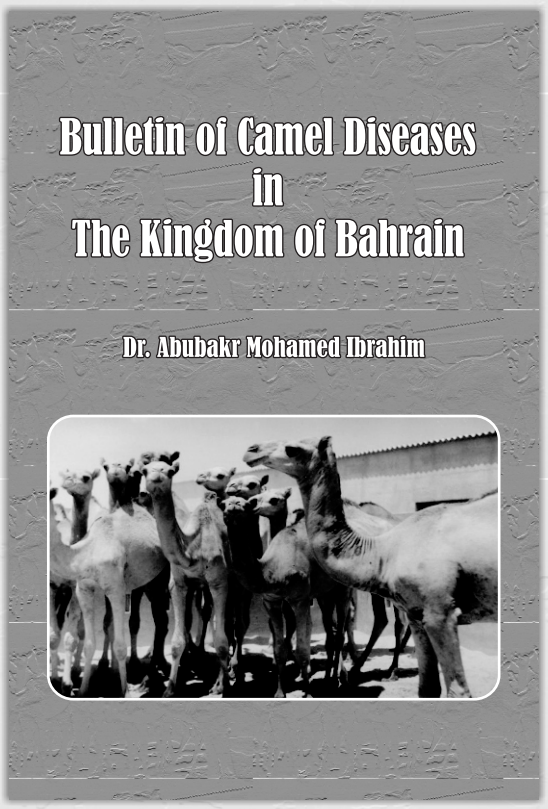
Bulletin of Camel Diseases in The Kingdom of Bahrain

This is a unique book which contains chapters on infectious and non-infectious diseases. The chapter on infectious diseases contains six sections. The section of bacterial diseases is subclassified as corynebacterium abscesses, paratuberculosis, hepatic necrobacillosis, mastitis, *Streptococcus zooepidemicus,* bacterial Infection in young camels, uterine Infection, infection of the vagina and vulva and other disorders. The section of protozoal diseases has narrations on trypanosomiasis, anaplasmosis and babesiosis. The section on parasitic infections is composed of gastrointestinal parasites in young camels, echinococcosis and mange. The section of mycotic diseases contains phycomycosis and ringworm. The section of viral diseases contains subsections on camel pox and contagious ecthyma. Edema Disease is described in miscellaneous section. The chapter on noninfectious diseases has three sections. Other section on poisoning describes pyrethroid, nitrate and toxic jaundice. The section describes zinc deficiency. The miscellaneous section describes foreign bodies, sand colic, bloat, caecal impaction, hydrocephalus, corneal opacity and osteochondroma.

About the Author

Dr. Abubakr Mohamed Ibrahim is a Veterinary Pathologist and worked for a long period as head of Royal Court Veterinary Laboratory. Kingdom of Bahrain which led to genesis of this publication out of his rich experience in diagnosing camel diseases in the Kingdom of Bahrain. This would be counted as his significant contribution and future researchers will find it easy to understand the pattern of camel diseases in this part of the world. Dr. Abubakr had majority of his publications based on camel diseases of Bahrain. Thus publication of this book would prove an important reference book for the camel practitioners and researchers.



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BACTERIOLOGICAL STUDIES IN CAMEL MASTITIS IN TAMBOUL AREA, SUDAN

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ABSTRACT

This study was carried out to identify the causative agents of different types of mastitis in Tamboul area. Sixty one milk samples from a field survey positive for change of colour using California mastitis test (CMT) were obtained from 10 herds comprising 1649 camels during 3 seasons (summer, winter and autumn) of same year. In the slaughter house investigation, 37 tissue samples and swabs obtained from different udder pathological lesions of 353 mastitis cases out of 2158 female camels of Arabi breed were tested by conventional bacteriological methods. The results revealed 98 isolates which consisted of 85 Gram-positive (86.73%) and 13 Gram-negative ones (13.27%). The isolates in order of frequency were *Staphylococcus* spp. (46.94%), *Streptococcus* spp. (18.37%), *Bacillus* spp. (13.27%), *Micrococcus* spp. (4.08%) and *Corynebacterium* spp. (4.08%). The Gram-negative bacteria isolates were *Escherichia coli* (8.16%), *Pseudomonas* spp. (4.0%) and *Salmonella typhimurium* (1.02%).

Key words: Bacteriological study, camel, mastitis, Sudan

Camel mastitis has been estimated to affect more than 25% of lactating she-camel (Saleh and Faye, 2011 and Alamin *et al,* 2013). Occurrence of mastitis in camels has been reported from some camel-keeping countries including Sudan (Obeid, 1983), Ethiopia (Regassa *et al,* 2013) and Egypt (Karmy, 1990). In Sudan the investigation of mastitis in the camel has been reported by various authors (Obied, 1983; Salwa, 1995; Nuha, 2001; Suheir, 2004; Yagoub, 2005; Alamin *et al,* 2013 and Abdella, 2015). Few studies indicate that some bacterial infections have been implicated as causes of mastitis in camels. This study was carried out to investigate the causes of mastitis in the herds at the field level and the female camels slaughtered at Tamboul slaughterhouse.

Materials and Methods

Ten herds from different parts of Butana area comprising 1649 she camels were examined in summer, winter and autumn during one year for change in milk using California mastitis test (CMT). Sixty one positive milk samples were taken aseptically into a sterile plastic container and placed in thermos flask containing ice for bacteriological examination. In addition, 37 tissue samples and swabs representing all types of pathological lesions encountered in 353 mastitis cases out of 2158 female camels of Arabian breed slaughtered at Tamboul slaughter house were taken for bacteriological examination. All these samples (98) were tested by conventional bacterial culture medium and other convenient media suitable for gram +ve and gram –ve bacteria (Barrow and Feltham, 1993).

Results

Tissues and swabs for bacteriological examination were taken from 37 udders representing all types of pathological lesions encountered in this study and 61 milk samples that were positive for CMT. These samples were tested by conventional bacteriological methods. All milk samples and lesions examined were positive for bacterial growth with the exception of one tissue lesion that didn't show any growth in media.

A total of 98 isolates were obtained from the samples of pathological lesions and milk (positive for CMT). They consisted of 85 Gram-positive isolates (86.73%) and 13 Gram-negative (13.27%).

The order of frequency of isolates was *Staphylococcus* spp. (46.94%), *Streptococcus* spp. (18.37%), *Bacillus* spp. (13.27%), *Micrococcus* spp. (4.08%) and *Corynebacterium* spp. (4.08%). The Gram-negative bacterial isolates were *Escherichia coli* (8.16%), *Pseudomonas* spp. (4.0%) and *Salmonella typhimurium* (1.02%).

Discussion

All these bacteria (Table 1 and 2) were isolated from affected udder and milk samples with the

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exception of *Micrococcus* spp. and S*almonella typhimurium* which were isolated from milk samples.

Table 1. Percentage of microorganisms isolated from pathological lesions.

Species	Total No. of isolates	%
Staphylococcus spp.	22	59.45
Bacillus spp.	6	16.22
Escherichia coli	4	10.81
Streptococcus spp.	2	5.41
Corynebacterium spp.	2	5.41
Pseudomonas spp.	1	2.70
Total	37	100%

Table 2. Percentage of microorganisms isolated from milk samples (positive for CMT) in the survey.

Species	Total No. of isolates	%
Staphylococcus spp.	24	39.34
Streptococcus spp.	16	26.23
Bacillus spp.	7	11.47
Micrococcus spp.	5	8.20
Corynebacterium spp.	3	4.92
Pseudomonas spp.	3	4.92
Escherichia coli	2	3.28
Salmonella typhimurium	1	1.64
Total	61	100%

In present study, the isolated Gram-positive bacteria constituted 86.7% of the total isolates. This is concordant with the findings of Hawari and Hassawi (2008), Hussein *et al* (2013), Wanjohi *et al* (2013) and Abdella (2015) who reported that Gram- positive cocci of the genera *Staphylococcus, Streptococcus* and *Micrococcus* were the most dominant udder pathogen isolated and were regarded as important pathogens in camel. The prevalence of Gram negative bacteria in the present study was lower than that given by Al-Tofaily and Alrodhan (2011), who reported 23.8% prevalence of *Salmonella, Klebsiella pneumonae* and *Mannheimia haemolytica.*

Micrococcus spp. was only isolated from milk positive for CMT. This result was similar to the findings of Al-juboori *et al* (2013) who reported 5% prevalence, but lower than that of Abdella (2015) who reported 8.11% prevalence.

Salmonella typhimurium represented 1.02% of the total bacterial isolates and 7.69% of the total Gram-negative bacteria isolated. This result was similar to that reported by Abdella (2015) and Al-Tofaily and Alrodhan (2011) who found only 2 isolates. *Salmonella typhimurium* is known to be hazardous to human health.

Escherichia coli represented 8.16% of the total bacterial isolates and 61.54% of the Gram- negative bacteria isolated. This is lower than the 18.9% prevalence reported previously (Abdella, 2015).

Pseudomonas spp. represented 4.08% of the total bacterial isolates and 30.77% of the Gram- negative bacteria isolated. *Pseudomonas* spp. were isolated from pathological lesions and milk samples. This finding constitutes the first record of the isolation of *Pseudomonas* spp. from mastitis of camels.

Escherichia coli and *Streptococcus* were the major infectious organisms in endometritis in camelids (Tibary *et al*, 2006). Some of these bacteria are part of the normal vaginal flora whereas others are opportunistic and can become pathogenic if the favourable conditions are present (Tibary and Anouassi, 2001).

The variations in types of organisms isolated indicate that camel environment are contaminated with organisms which tends to flourish under stressful condition.

The predominant isolated organisms associated with clinical mastitis in the survey were *Staphylococcus* spp. (39.34%). This result agrees with the studies of Abdella (2015) who reported that the *Staphylococcus* spp. was 37.8%. This result was similar to that reported by percentage by Saleh and Faye (2011) and Hussein *et al* (2013) who reported that the *Staphylococcus* spp. was 42.9% and 43.8%, respectively. Alamin *et al* (2013) reported that 80.3% of she-camels examined suffered from wounds on the teats caused by pieces of wood and cloth used in the anti-suckling devices. *Staphylococcus* spp. might spread between she-camels due to these anti-suckling devices. In this study *Staphylococcus aureus* has been identified as the most commonly isolated (54.34%) *Staphylococcus* spp. that causes mastitis.

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Camel milk slowly gaining attention of health-conscious residents of UAE

The price of camel milk may be twice as much as traditional cow milk in the UAE market, but it is slowly gaining attention of health-conscious residents for its nutrient-rich features. "One litre of cow milk is around Dh6 but camel milk will cost you Dh20. It is quite expensive since camels produce much less milk on a daily basis than cows. But the benefits camel milk provides outweigh the price," Haider Shah, general manager of Café2go, told Khaleej Times. The coffee shop's brand, Camellos, serves snacks and beverages with camel meat and milk as a prime ingredient.

Mutasher Awad Al Badry, deputy general manager and business development manager of Emirates Industry for Camel Milk and Products says that camel milk production is less than cow milk production. Further, camel breeding costs are more than cows and fact remains that cows produce milk over the year, but with camels, the cycle is repeated once every three years. Therefore, the cost of a litre of camel milk may be costing equal to three litres of cow's milk.

Camel: The new cash cow

A general report by Euromonitor titled 'Dairy in the UAE' reveals that drinking milk products recorded a value CAGR (Compound Annual Growth Rate) of two per cent in 2016 and are expected to reach sales of Dh1.6 billion in 2021. This is largely in line with the corresponding CAGR recorded over the review period, reflecting the maturity of the category as well as the impact of strong price controls on its products.

Consumers lack information about the health benefits that camel milk offers. Camel milk has 10 times more iron and three times more vitamin C than cow's milk. Additionally, camel's milk is lower in cholesterol, fat and high in protein. There have been medical research showing that camel milk has actually helped children with autism live a better life, and individuals who are lactose intolerant are able to cope with camel milk much better. To date, camel milk is available in original as well as flavoured varieties - such as chocolate, strawberry and dates - to appeal to demanding tastebuds. It is further blended into other appetising treats such as laban, cheese, and even ice cream.

(The Khaleejtimes: Camel: The new cash cow, March 2017).

Special session on camels in 11th International Veterinary Congress at Berlin, Germany

Special session on camels with a theme "Camel Research: Challenges and Opportunities" will take place in 11th International Veterinary Congress at Berlin, Germany scheduled on 2-3 July 2018.

There will be limited participants in this special session and their deliberations will be of great value to the participants. Desirous participants are requested to submit their abstracts at an earliest.

Dr. T.K. Gahlot is the Organizing Secretary for the special session and he can be contacted on email tkcamelvet@yahoo.com

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DEMONSTRATION OF HEPATOPROTECTIVE ACTION OF CAMEL MILK THROUGH IMPROVING ANTIOXIDANT ACTIVITY AND REGULATING GENE EXPRESSION IN MICE

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ABSTRACT

The composition of camel milk includes antioxidants that are beneficial for liver function and regulate gene expression. Here we evaluated the protective effects of camel milk in mice. Our results revealed that camel milk protected the liver by decreasing levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and malondialdehyde (MDA), and increasing the activity of superoxide dismutase (SOD) and glutathione (GSH) in mice. Histopathological examination demonstrated that the livers of mice receiving camel milk were not different to those of control mice. The mRNA expression of SAA1, TGF-α, TNF-α, and LCN2 was down-regulated in the livers of mice receiving camel milk. These results indicate that camel milk significantly increases the antioxidant capability of the liver and regulates gene expression in mice. Thus it is opined that regular consumption of camel milk has hepato-protective effect.

Key words: Camel milk, Hepatoprotective, Antioxidant activity, Regulating gene expression

The liver plays a key role in the metabolism of foreign compounds entering the body; contact with polluted environments, consumption of contaminated food and exposure to toxic chemicals can lead to a variety of different types of liver disease in humans (Rajesh and Latha, 2017). In recent years, there has been much research into the therapeutic effects of camel milk for the amelioration of liver disease symptoms and the side effects of treatments. For example, El-Bahr (2014) reported that camel milk protected the liver against carbon tetrachloride (CCl_4)-induced hepatic toxicity by modulating the extent of lipid peroxidation and by boosting the antioxidant defense system both at the activity and the gene expression level. Darwish *et al* (2012) found that treatment with camel milk alleviated alcohol-associated liver disfunction and protected hepatic tissues from alcohol-induced toxicity. Regular consumption of camel milk could also provide a natural way to protect against non-alcoholic fatty liver disease induced by a high-fat diet (Korish *et al,* 2013), and halt the progression of hepatocellular carcinoma (Miniawy *et al,* 2014). Overall, camel milk contains factors that have the potential to be protective and therapeutic in the liver.

Camel milk has a wide range of antioxidative, antimicrobial and immuno-modulatory properties (Mihic *et al,* 2016). In view of this, the present study was done to demonstrate the hepato-protective effects of camel milk on the regulation of gene expression and activity of hepatic antioxidant enzymes in mice.

Materials and Methods

Collection and processing of camel milk. Milk was collected from 35 camels (Alxa League, Inner Mongolia, China), stored in sterilised containers on ice, and immediately transported to the laboratory. Once in the laboratory the milk was frozen at -80°C prior to pasteurisation at 65°C for 30 minutes and then freeze-dried into milk powder. The milk powder was pressed into pellets for long-term storage at -80°C prior to use in experiments. During experiments the milk powder was reconstituted with sterile water (dry matter was 15%) in a sterile environment.

Animal treatments. Twenty male C57BL/6J mice (12 weeks old) were housed in a room maintained

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under standard laboratory conditions (12 h light/dark cycle; temperature of 21-23°C; relative humidity of 45-65%). Mice had free access to sterilised standard chow and distilled water throughout the experiment. The animals received humane care and all protocols were approved by Animal Care and Use Committee at Inner Mongolia Agricultural University.

After acclimatisation to standard laboratory conditions for 7 days, mice were randomly assigned to two groups (10 per group) as follows:

Control group (C) received sterile distilled water (10 ml/kg body weight/day) intra-gastrically.

Camel milk group (M) received camel milk (10 ml/kg body weight/day) intra-gastrically.

After 4 weeks, mice were euthanised using isoflurane gas. Blood samples were collected into heparin-containing blood sampling tubes, centrifuged at 2500 g for 15 min at 4°C and the supernatant transferred to a clean tube and frozen at -80°C until further analysis. The liver of each mouse was dissected out quickly. A portion of the left lobe of each liver was excised and fixed in a 4% paraformaldehyde solution for histopathological analysis.

Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum. In order to evaluate the liver-protection capacity of camel milk, the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice receiving camel milk were compared with control mice. The levels of ALT and AST in mouse serum were determined using commercial assay kits (Roche Diagnostics, Switzerland) according to the manufacturer's protocols.

Determination of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) levels in the liver. After homogenisation and centrifugation, the supernatants of liver tissues were evaluated for the activity of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) by ELISA (Sangon Biotech Co., Ltd., Shanghai, China). Results were calculated by following the manufacturer's instructions. This was because MDA level is widely used as a marker for free radical mediated lipid peroxidation injury and SOD and GSH are used as indices for the antioxidant status of tissues.

Quantitative RT-PCR Analysis. Liver tissue (50-100 mg/ mouse) was homogenised in 1 mL of TriZol reagent (Invitrogen) and total RNA was extracted. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed as follows: cDNA was synthesised with a ReverTra Ace-a Kit (Toyobo) from total RNA after DNase I treatment, and real-time PCR was performed using a QuantiFast SYBR Green PCR Kit (Qiagen). Every plate included the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control. Particular interest in the expression of genes for cancer-associated factors and inflammatory factors was kept in mind. The primer sequences used for each gene are listed in Table 1. The $2^{-(\Delta\,\Delta Ct)}$ method was used to calculate the results.

Histopathological examination. The fixed liver tissue samples (4% paraformaldehyde, 4°C) were embedded in paraffin, cut into 5 µm sections and stained for histopathological examination.

Statistical analysis. Unless otherwise indicated, all values are expressed as the mean ± SEM. Statistical analyses were performed using Student's t test. Statistically significant differences between groups were defined as $p < 0.05$.

Results

Effects of camel milk on AST and ALT levels in serum

Levels of ALT ($P<0.01$) and AST ($P<0.01$) were significantly lower in the mice receiving camel milk compared to the control mice (Fig 1).

Effects of camel milk on MDA, SOD and GSH levels in liver tissue

The levels of MDA were lower in the livers of mice receiving camel milk compared with control mice, although this was not statistically significant ($P>0.05$) (Table 2). The levels of SOD ($P<0.05$) and GSH ($P<0.05$) increased significantly in the livers of mice receiving camel milk compared with control mice (Table 2).

Quantitative RT-PCR Analysis

The mRNA expression of serum amyloid A 1 (SAA1) was significantly down-regulated in the livers of mice receiving camel milk compared with control mice (Fig 2A, $P<0.01$). This was also the case for transforming growth factor-α (TGF-α) (Fig 2B, $P<0.01$), tumor necrosis factor-α (TNF-α) (Fig 2C, $P<0.01$) and lipocalin 2 (LCN2) (Fig 2D, $P<0.01$).

Histopathological examination

Histopathological changes in the liver are shown in Fig 3. In normal control animals, gross macroscopic evaluation of the liver showed red,

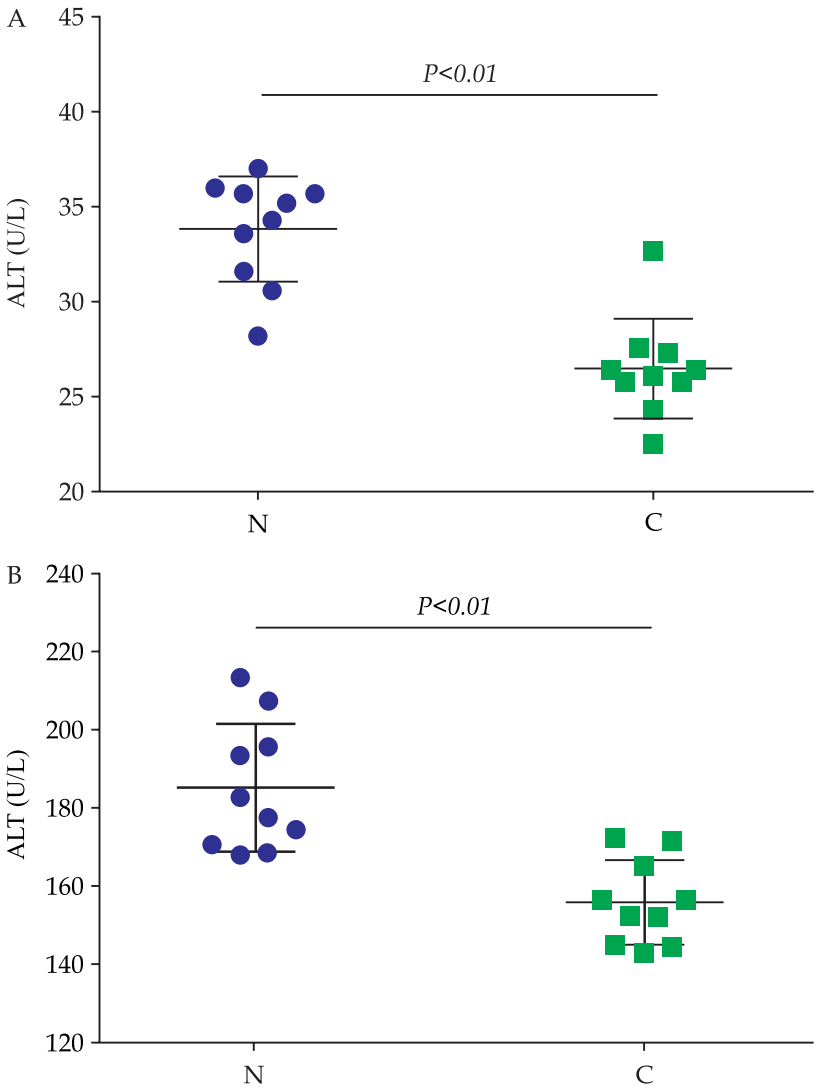

Fig 1. Effects of camel milk on serum AST and ALT activities.

smooth and shiny liver tissues (Fig 3A), and liver sections showed normal hepatic cells with well preserved cytoplasm, prominent nucleus and nucleolus and central vein (Fig 3C). Liver gross macroscopic and sections of the mice receiving camel milk group were similar to the control group mice (Fig 3B and 3D).

Discussion

Camel milk is widely used in various populations for the treatment and prevention of diseases (Yagil, 2013). Our results revealed that camel milk could protect liver by decreased levels of MDA, and increase activities of SOD and GSH in mice. An increase in the levels of MDA in the liver enhances peroxidation and can lead to tissue damage and failure of the antioxidant defence mechanisms that prevent formation of excessive free radicals (Sun *et al,* 2013). Consumption of camel milk is known to have beneficial antioxidative properties in the treatment of many diseases, and also that it inhibits lipid peroxidation (MDA) in mice (Mihic *et al,* 2016). Our findings are in accordance with these previous studies.

GSH, is the most abundant thiol in mammals, was discovered a century ago, and has a central function in the detoxification and protection of oxidants (Li *et al,* 2012). GSH is an extremely efficient intracellular buffer for oxidative stress (Hsu *et al,* 2008). Furthermore, SOD can also reduce oxidative stress, and is an effective defense enzyme that converts the dismutation of superoxide anions into hydrogen peroxide (Li *et al,* 2012). Several studies have found that antioxidant enzymes such as SOD and GSH provide protection against oxidative tissue-damage (Hsu *et al,* 2008). We found that the activity of GSH and SOD in liver tissue of mice receiving camel milk increased significantly compared with control mice and suggest that these two enzymes contribute to the hepato-protective effects of camel milk in mice.

The liver is an important organ of the human body, and internal mechanisms for protection of the liver are the main way that damage to liver cells is prevented. Serum amyloid A (SAA) is a pro-inflammatory molecule that induces leukocyte infiltration and promotes neutrophil adhesion to endothelial cells under inflammatory conditions (Young *et al,* 2015). In a case study of a patient with alcoholic liver cirrhosis, an existing liver nodule was diagnosed as SAA-positive by immunohistochemistry (Kim *et al,* 2014). SAA1 is an isoform of SAA that has been reported in mice (Young *et al,* 2015). SAA1 increases expression of AST and ALT as well regulating secretion of pro-inflammatory cytokines during hepatitis (Young *et al,* 2015). In our study, the expression of SAA1 was down-regulated in the livers of mice receiving camel milk, thus contributing to protection of the liver from inflammation.

TGF-α is a cellular factor that plays a role in regulation of healthy and tumour cell proliferation

Table 1. Primer sequences used for quantitative RT-PCR.

Gene	Sense	Anti-sense
SAA1	CAGCTACCAATCAGGCATGTC	ATGTCTGCTCGAAGCATTAAC
TGF-α	CTGGCTGTCCTCATTATCACCT	AAATTCCTCCTCTGGGATCTTC
TNF-α	AAGCCTGTAGCCCACGTCGT	CGTAGTCGGGGCAGCCTTGTC
LCN2	AAAGACCCGCAAAAGATGTATG	AACCTGGAACAAAAGTCCTGAT
GAPDH	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC

Table 2. Effects of camel milk on MDA, SOD and GSH levels in mouse liver.

Group	MDA(nmol/g)	SOD(U/g)	GSH(ng/g)
N	120.193±10.123	1752.153±84.678	52.661±3.162
C	115.119±8.265	1816.094±78.356*	55.574±2.406*

Each value represents the mean±SD. * p<0.05, compared with N group.

and differentiation (Koshibu and Levitt, 2005). In human cancers, studies have shown that TGF-α could serve as a tumour marker and as a marker for the malignant potential of a tumour (Grigioni, 2002). To date, the types of carcinomas with which abnormal TGF-α expression has been associated include liver, breast and ovarian cancers (Zhang *et al,* 2004). Our result showed that the expression of TGF-α was down-regulated in mice receiving camel milk and this could explain why consumption of camel milk can halt the progression of hepatocellular carcinoma.

Liver injury is reportedly associated with a chronic inflammatory response involving TNF-α (Farinati *et al,* 2006). Hepatocyte apoptosis induced by TNF-α is a common pathological phenomenon and the mechanism driving many liver pathologies in the early stage. TNF-α is associated with NAFLD and induced inflammatory cytokines formation (Tilg *et al,* 2011). More importantly, TNF-α-mediated hepatocyte injury not only leads directly to hepatocyte necrosis, but also mediates apoptosis (Guicciardi *et al,* 2001). Our result showed that the expression of TNF-α was down-

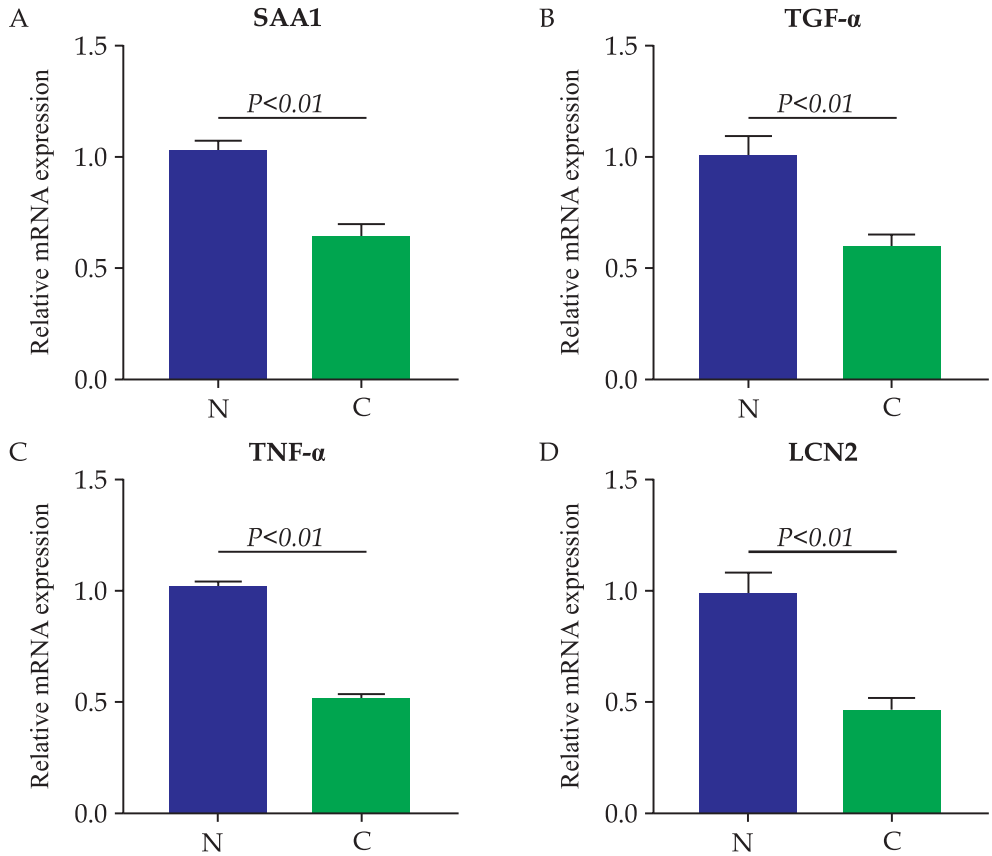

Fig 2. Relative mRNA expressions of SAA1, TGF-α, TNF-α and LCN2 in mouse liver.

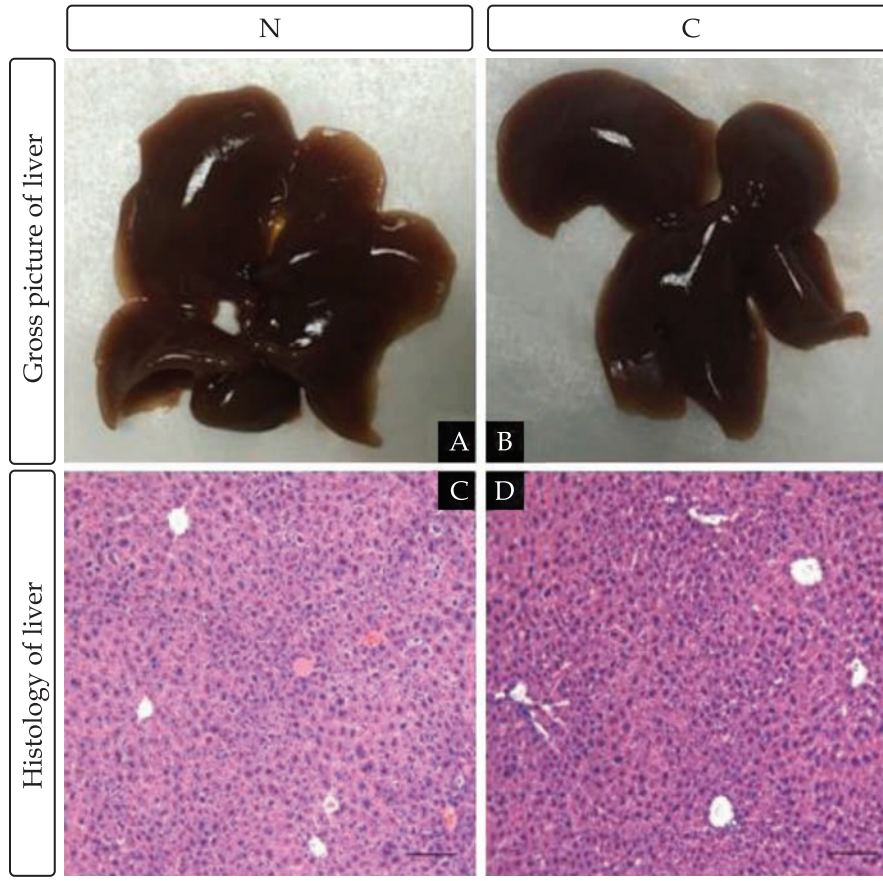

Fig 3. Gross and histology changes in the liver from normal group and camel milk group mice. HE staining (20X).

regulated in the livers of mice receiving camels milk, again explains hepato-protective action of camels milk.

The protein LCN2 is a secretory glycoprotein that is secreted by liver cells and may play a protective role when stimulated by inflammatory factors; it is up-regulated during many cellular stresses (Zhang *et al,* 2014). There is increasing evidence that LCN2 plays a protective role in liver injury. Wan *et al* (2017) found that inhibition of inflammation by curcumin was related to LCN2 down-regulation. We found down-regulation in the expression of LCN2 in the livers of mice receiving camel milk, showing that camel milk can protect liver cells.

Our results in mice have revealed that camel milk treatment could protect the liver by decreasing the levels of ALT, AST and MDA, increasing SOD and GSH activity and down-regulating mRNA expression of SAA1, TGF-α, TNF-α and LCN2. Thus, regular consumption of camel milk could increase the antioxidant capacity of the liver and regulate gene expression; in this way camel milk can prevent the liver from being damaged. Camel milk is a potential liver-protective food without any side effects.

In conclusion, this study has indicated the protective effect of camel milk in mice. The mechanism for liver protection was restoration/ enhancement of the activities of antioxidant enzymes and the inhibition of lipid peroxidation. By enhancing the antioxidant ability of hepatocytes camel milk decreased various toxic substance-induced oxidative stresses in the liver. Further studies with individual active compounds that exist in camel milk are underway which will enable us to understand the exact mechanisms responsible for liver protection.

Acknowledgements

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Ethical Approval

All animal procedures were approved by Animal Care and Use Committee at Inner Mongolia Agricultural University.

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STAPHYLOCOCCAL SUBCLINICAL MASTITIS IN DROMEDARY DAIRY CAMEL

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ABSTRACT

Camel milk samples were randomly and aseptically collected after discarding first milk streak from 243 quarters of milking camels without clinical sign of mastitis or bacteriological investigation. There was no bacterial isolation in 61.73% samples. The bacteria isolated in remaining samples were *Staphylococci* (n=78; coagulase negative Staphylococci: 71 and *Staph. aureus:* 7) and *Streptococci* (n=26). Five new species of coagulase negative Staphylococci including *Staph. saprophyticus, Staph. delphini, Staph. capitis, Staph. chromogenes* and *Staph. caseolyticus* were identified. PFGE analysis carried out on 7 isolates of *Staph. aureus* and produced 2 distinct pulsotypes designated as pulsotypes A and B. All *Staph. aureus* isolates were found to be included into 2 spa types: t527 and t1532. Coagulase negative staphylococci were the main bacteria isolates (55.04%) and the frequency of *Staph. aureus* with 2 spa types was considered as 5.43% of total bacterial isolation.

Key words: Dairy dromedary camel, *S. aureus*, subclinical mastitis

Prevalence of clinical mastitis in camel was assumed to be low (Manefield and Tinson, 1996; Wernery and Kaaden, 2002). However, by looking at the microflora involved in teat canals and udder cisterns in non-lactating dromedary camels (Johnson *et al,* 2015a), it is expected that machine milking may predispose lactating camels to subclinical mastitis, similar to other milk-producing animals.

Various species of bacteria have been found as the main cause of mastitis in camels including *Staph. aureus*, *Strep. agalactiae,* Coagulase Negative Staphylococci (CNS), *Strep. bovis*, *Escherichia coli*, *Micrococcus* spp., *Corynebacterium* spp. and *Aerobacter* spp. (Barbour *et al,* 1985; Abdurahman, 1995; Obeid *et al,* 1996; Wernery *et al,* 2008; Johnson *et al,* 2015b). Some *Staph. aureus* isolates are drug resistant and are conserved as public health threat (Fitzgerlad, 2012; Ansari *et al,* 2014). As a result, livestock-originated *Staph. aureus* is of significance in terms of human health and are required to be further characterised (Fitzgerlad, 2012). Saleh and Faye (2011) found *S. aureus* and other species of staphylococci as main causative agent for subclinical mastitis in dromedaries.

The objective of this study was to investigate the frequency and type of bacteria causing subclinical mastitis and to find the possible genotypes of principle isolate from milk samples of dromedary camels.

Materials and Methods

Experimental design

Camel milk samples were collected from 243 quarters of milking camels (7-11 years of age, 2-4 months after calving), without any observable disease and clinical signs of mastitis. Prior to sampling, the teat was washed and the camel calf was released to stimulate milk let down from the dam. Immediately after teat engorgement, the suckling was interrupted and teats were disinfected with cotton moistened with 70% alcohol. After discarding the first few squirts of milk, about 50 ml of milk were collected into sterile bottle and kept cool these were transported to the laboratory for bacteriological investigations.

Bacteriological investigation

Bacteriological isolation was conducted according to the standard procedure (MacFaddin,

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2000). In brief, milk sample (30-50 µL) was streaked on 5% sheep blood agar (Merck, Germany) and MacConkey agar (Merck, Germany). Plates were incubated at 37ºC for 5 days. Presumptive identification of bacteria on primary culture was conducted according to the morphology of colony, haemolytic characteristics, Gram stain and catalase test. *Staphylococci* were identified based on coagulase test. CNS were identified using classic chemical procedure (MacFaddin, 2000). *Streptococci* isolates were evaluated based on CAMP reaction, aesculin hydrolysis test and growth on 6.5% sodium chloride (MacFaddin, 2000). Gram-negative isolates were further tested using triple sugar iron (TSI), urease, indole, MRVP, citrate and lysine decarboxylase tests.

Isolation of Staphylococcvs aureus

The species of isolated *S. aureus* was identified using standard biochemical methods including gram staining, catalase, DNase, fermentation of mannitol and production of coagulase. To confirm the identity of the species, the nuc gene was amplified by a PCR-based method, using the specific primers (nucA-F 5′-CTGGCATATGTATGGCAATTGTT-3′ and nucA-R 5′-ATTGACCTGAATCAGCGTTGTCT-3′).

PFGE analysis

The entire genomic DNA was prepared as described previously (Fatholahzadeh *et al,* 2009). After digestion with *SmaI* endonuclease, the DNAs were separated by CHEF electrophoresis system (AP-Zoha Ltd, Tehran, Iran) for 20 h at 14ºC with an electric field of 6 V/cm in 0.5× TBE buffer. The pulse time increased from 1 to 30 s (10 h) and 1 to 3 s (10 h). The gels were stained with ethidium bromide (1µg/ml) and visualised by UV illumination. A reference strain of *S. aureus* (NCTC8325) was prepared in the same way and run as the molecular size standard. The assessment and interpretation of PFGE patterns were performed visually according to the defined criteria (Tenover *et al,* 1995).

SPA typing

The spa typing was performed according to the method described previously (Emaneini *et al,* 2011) followed by amplification and sequencing of the spa gene X region. PCR reactions were performed in a 50 µL volume consisting of 1X PCR buffer, 3 mM $MgCl_2$, and 0.4 µg/ml of each primer, 1.5 U Taq DNA polymerase, 0.2 mM dNTP Mix and 5 µl of DNA template. The PCR conditions consisted of a pre-denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 40 s, 55 °C for 40 s and 72 °C for 60 s. A final extension step was performed at 72 °C for 10 min. Sequences of both strands of the amplicons were determined at Macrogen (Seoul, South Korea). Isolates were assigned to particular spa types according to the guidelines described by the spa typing website (http://www.spaserver.ridom.de).

Results and Discussion

In 150 quarter milk samples (61.7%), no bacteria were isolated. Accordingly, quarter infection rate was 38.3% (93/243). Out of 129 bacteria isolated from 93 quarter milk samples, *Staphyloccoci* was isolated from 78 cases (60.46%), out of which 7 cases (8.97%) were *S. aureus* and the rest (71; 91.03%) were CNS (Table 1). CNS bacteria had a varied prevalence, i.e. *S. saprophyticus* (47.44%), *S. schleiferi* (16.67%) and *S. epidermidis* (14.10%). *Streptoccoci* were isolated from 26 cases (20.16%), out of which *Strep. uberis* (7.75%) and *Strep. mutans* (5.43%) had greater incidence (Table 1). The rest of bacteriological isolation was dedicated to coliforms (7.75%), *Pseudomonas* (5.43%),

Table 1. Mixed and pure bacteria isolation from quarter milk samples of dromedary camel.

Bacteria	Total	No. in Genus	Pure isolation	Mixed isolation	% in Genus	% in Total
Isolated bacteria	129					
Staphylococci	78					60.46
Staph. aureus		7			8.97	5.43
Coagulase Negative Staphylococci		71			91.03	55.04
Staph. saprophyticus		37	19	18	47.44	28.68
Staph. schleiferi		13	9	4	16.67	10.08
Staph. epidermidis		11	4	7	14.10	08.53
Staph. delphini		4	2	2	5.13	03.10
Staph. capitis		3	1	2	3.85	02.32
Staph. chromogenes		2	1	1	2.56	01.55
Staph. caseolyticus		1	1	0	1.28	0.77
Streptococci	26					20.16
Strep.uberis		10	2	8	38.46	07.75
Strep. mutans		7	1	6	26.92	5.43
Strep. agalactiae		5	2	3	19.23	3.87
Strep. alactolyticus		3	2	1	11.54	2.32
Strep. dysgalactiae		1	1	0	3.85	0.77
Coliform	10		1	9		7.75
Pseudomonas	7		1	6		5.43
Corynebacterium	6		3	3		4.65
Proteus	2		2	0		1.55

Corynebacterium (4.65%) and *Proteus* (1.55%; Table 1). There were no *E. coli* or pathogenic corynebateria isolated from the milk samples.

PFGE analysis of the 7 *S. aureus* isolates produced 2 distinct pulsotypes designated as pulsotypes A and B. All *S. aureus* isolates were found to be included into 2 spa types: t527 and t1532 (Table 2).

In the present study, no bacteria were isolated from 61.7% of milk samples (150/243), similar to the result reported from Israel (60.6%; 83/137; Chaffer *et al,* 2000) and UAE (65.87%; 195/297; Johnson *et al,* 2015b). Similar results were reported by in non-lactating dromedary camel after collecting swabs from teat canal (76%; 184/242) and cistern (79%; 189/242). This condition occurs in dairy cows with the prevalence of 30% (Philpot and Stephen, 2000). The low number of bacteria isolated from camel milk might be unique for this species. It could be explained by numerous antimicrobial agents in camel milk such as lysozyme, lactoferrin, lactoperoxydase and immunoglobulins that could limit microbial growth to higher degree than in milk from other domestic animals (Korhonen and Pihlanto, 2006; El-Hatmi *et al,* 2007; Salami *et al,* 2010).

Table 2. PFGE analysis and spa typing of 7 *S. aureus* isolates.

Isolation	PFGE	SPA Typing
17	A	t527
28	A	t527
39	A	t527
41	A	t527
167	B	t1532
174	B	t1532
220	B	t1532

Based on isolation of bacteria, quarter infection rate was 38.3% (93/243) in the present study. Several studies with great variation were reported to illustrate the prevalence of subclinical mastitis in dromedary camel, i.e. 36.87% (59/160 camels; Suheir *et al,* 2005), 15% (9/60 quarters; Alamin *et al,* 2013) in Sudan, 15.8% (80/505 quarters; Abera *et al,* 2010) and 20.7% (30/145 camels; Abera *et al,* 2010), 22% (43/195 camels, Almaw and Molla, 2000), 67.4% (433/642 quarters; Seifu and Tafesse, 2010), 39.4 % (137/348 camels, Regassa *et al,* 2013) in Ethiopia, 11.67% (21 /180 camels; Ibrahim *et al,* 2011) in Saudi Arabia, 38% (57/150 camels; Sibtain *et al,* 2012) in Pakistan and 41% (41/100 quarters) and 72% (18/25 camels) in India (Bhatt *et al,* 2004). Accordingly, it may be concluded that the prevalence of subclinical mastitis in camel could be within the range of 11-72% on specieswise and 15-67% on quarter basis, providing an indication that subclinical mastitis may be considered as existing problem in dairy dromedary camel.

The majority of isolates in the present study were of *Staphylococci* spp (60.46%) and *Streptococci* spp (20.16%). It was in consonance with previous investigations in Kenya (Wanjohi *et al,* 2013), Ethiopia (Woubit *et al,* 2001; Abera *et al,* 2010), Sudan (Alamin *et al,* 2013), Pakistan (Sibtain *et al,* 2012), India (Bhatt *et al,* 2004) and Israel (Chaffer *et al,* 2000). Among *Staphylococci* in the present study, CNS were isolated in the majority of cases (55.04%). CNS prevalence in camel was 46% in Iraq (Almaw and Molla, 2000), 20.4% in Israel (Chaffer *et al,* 2000) and 7.5% in Ethiopia (Abera *et al,* 2010). In the present study new species of CNS bacteria were identified including *S. saprophyticus*, *S. schleiferi*, *S. delphini*, *S. capitis*, *S. chromogenes* and *S. caseolyticus*. It was only *S. epidermis* that has been previously reported (Obeid *et al,* 1996; Suheir *et al,* 2005). *Staphylococci* are common inhabitants of the skin and the mucosal surfaces of humans and various animals. CNS constitutes part of the physiological flora (Werckenthin *et al,* 2001; Adegoke and Okoh, 2014). Most *staphylococcal* species are considered as facultative pathogen. The pathogenic capacity of CNS mainly depends on whether they possess virulence genes (Oogai *et al,* 2011). On the host side, the intact skin or mucosal surface represents the first mechanical barrier against these infectious agents. In addition, a functionally active host immune system as well as the tissue-specific commensal flora play important role as biological barriers against pathogenic bacteria, including *staphylococci* (Singh and Morris, 2012). Open injuries, burns, scratch and bite wounds and primary viral or parasitic infections, which could destroy this mechanical barrier, enable *Staphylococci* as inhabitants of the skin or the mucosa to reach deeper tissues and cause an either localised or generalised infection (Werckenthin *et al,* 2001). The latter happens mainly when the *staphylococci* disseminate *via* blood and can evade or suppress the host's immune system. Certain virulence factors of *Staph. aureus*, in particular the Panton-Valentine leukocidin, target and destroy components of the host immune system (Holzinger *et al,* 2012). Intramammary infections caused by CNS are common in dairy cows as well (Thorberg *et al,* 2009). In comparison with *S. aureus*, CNS has lower pathogenicity, but they could have an important role in subclinical mastitis and elevation of SCC in ruminants (Contreras *et al,* 2007; Thorberg *et al,* 2009). The prevalence of *S. aureus* was relatively low (5.43%) in the present study, similar to the report from Ethiopia (2.9 %, 39/1362; Regassa *et*

al, 2013), Israel (8.8%; Chaffer *et al,* 2000) and Sudan (5.4%; Abdurahman *et al,* 1995). In other studies, *S. aureus* was considered as the main cause of subclinical mastitis in camel in Saudi Arabia (27.91%; Ibrahim *et al,* 2011), Ethiopia (26.3%; Abera *et al,* 2010), and Sudan (20.2%; Suheir *et al,* 2005).

In this study the molecular characteristics of *S. aureus* was elucidated. There is few data about the genetic analysis of *S. aureus* isolated from camel milk (Shuiep *et al,* 2009; Monecke *et al,* 2011; Jaradat *et al,* 2014). Further studies from various geographical regions are needed to investigate the molecular characteristics of *S. aureus* by various typing methods and to determine the genetic nature of this bacterium. A variety of genotyping techniques are available for classifying *S. aureus* strains for epidemiological investigation, including band-based (e.g., PFGE) and sequence-based methods (e.g., MLST, spa typing). PFGE is the gold standard for typing of *S. aureus* strains and is known to be a highly discriminatory and valuable technique for outbreak investigation (Tenover *et al,* 1995; Eslampour *et al,* 2009). Nevertheless, spa typing contains significant advantages over PFGE such as ease of use, reproducibility, transportability and comparability of the results (Emaneini *et al,* 2011). To the best of our knowledge, this is the first study reporting the molecular characteristics of *S. aureus* isolated from camel milk by PFGE and Spa A typing methods. Results of the present study showed that all *S. aureus* isolates were of 2 genotypes. The presence of 2 genotypes of *S. aureus* might be the result of its increased resistance to the host immune response. Spa type t527 collected from different locations in Croatia, and in MRSA isolates of human and bovine samples in Iran. Spa type t1532 was found in 5 isolates from different locations in France and Portugal (http://www.spaserver.ridom.de). Concordance between PFGE results and spa typing technique in our study was 100%. Koreen *et al* (2004) showed that cross-classification concordance results between PFGE and spa typing was 98%.

The isolated *Streptococci* spp. in the present study were *Strep. uberis, Strep. mutans, Strep. alactolyticus, Strep. agalactiae* and *Strep. dysgalactiae.* Mastitis caused by *Strep. agalactiae* is prevalent in camel and has been reported in UAE (Quandil, 1984), Egypt (Karamy, 1990), India (Younan *et al,* 2001), Ethiopia (Almaw and Molla, 2000), Kenya (Younan *et al,* 2001) and Sudan (Abdurahman *et al,* 1995; Obied *et al,* 1996). Subclinical mastitis caused by *Strep. agalactiae* consisted of up to 74.4% and 50% of all cases of subclinical mastitis in India and Kenya, respectively (Younan *et al,* 2001). In addition, clinical mastitis due to *Strep. agalactiae* has been reported in Ethiopia (Almaw and Molla, 2000).

Taken together, the prevalence of subclinical mastitis was relatively low in the present study. It is well known that susceptibility to mastitis is determined by a combination of factors including bacterial virulence, environmental conditions (housing, management, feeding and milking technique) and animal-related factors (milk yield, genetics). These factors are interdependent to each other and their impact depends on the type of pathogen (Burvenich *et al*, 2003). The streak canal is relatively thin in camel which could play a role in low prevalence of mastitis in this species (Manefield and Tinson, 1996). Moreover, the cover used to prevent the calf from suckling has been suggested as a reason for low rate of mastitis in camel (Manefield and Tinson, 1996; Wernery and Kaaden, 2002). It is believed that the cover protect the animal from mechanical traumas. Yet it should be considered that the cover could be moistened with milk and become contaminated with bedding, and consequently predispose the animal to intra-mammary infections. Nevertheless, no research has been conducted in this regard, any hypothesis requires to be tested by a well-designed controlled study. In addition, machine milking is uncommon in camel, which might have contributed as additional reason for low prevalence of mastitis in this species. The other suggested factors for low prevalence of mastitis in camel are the type of resting, few contact of mammary glands with the bedding, low density of animals in the pasture and the dryness of bedding. Finally, one of the main potential factors in this context is the antimicrobial components of camel milk (Korhonen and Pihlanto, 2006; El-Hatmi *et al,* 2007; Salami *et al,* 2010). Further studies are warranted to investigate the underlying mechanisms for low prevalence of mastitis in camel.

In conclusion, *Staphylococci*, particularly coagulase negative staphylococci were the most frequent bacteria isolated from camel milk. Five new species of CNS were identified and *S. aureus* was characterised into 2 genotypes in camel milk samples.

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RECOLLECTION: CAMEL MILK PROTEINS, BIOACTIVE PEPTIDES AND CASEIN MICELLES

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ABSTRACT

Camel milk is mostly known through its unique proteins and bioactive peptides. Its protein pattern is more close to human milk than bovine milk. Camel milk similar to human milk lacks β-lactoglobulin, the most allergen protein in bovine milk. Bioactive peptides produced from both casein and whey proteins of camel milk have shown great bioactivity compared to peptides from different sources. Camel milk bioactive peptides have shown high antioxidant activity, ACE inhibitory activity as well as antimicrobial activity. The casein micelles of camel milk are an excellent nano carrier for bioactive components. Therefore, camel milk is considered as a new traditional superfood. Traditionally, it has been used since ancient times in some part of the world and it has been noticed as a new source for producing superfood in developed countries. Consuming milk and milk products is part of a healthy diet. Commercial milk is mostly bovine milk which has become one of the main sources of producing functional products through fortification and enrichments. Investment on camel milk has earned a good attention in today's food industries. In this recollection, we have pointed out some of the most important and recent works examined on camel milk and its components by our laboratory.

Key words: Bioactive peptides, camel milk, casein micelles, milk proteins, nutraceuticals, superfood

Commercial milk is mostly derived from bovine origin, which has become one of the main sources of producing functional products through fortification and enrichments. Camel milk is a nutritious food, consumed traditionally in some parts of the world. More recently, camel milk was considered as a superfood in developed countries as well. Because of similarities between camel and human milk, camel milk has earned a good attention in today's food industries. The objective of this recollection was to elaborate some of the recent works conducted in our laboratory on protein content, bioactive peptides and casein micelles derived from camel milk. Nowadays, the focus of food industry is on the production of functional, nutraceutical and or medicinal foods by fortifying and enriching the available source of food such as milk. Camel milk is a natural enriched product without any particular additives. Camel milk differs from bovine milk in composition and structure of its protein, vitamins and minerals, which influences its functional and biological properties. Camel milk composition is much closer to human milk than that of bovine milk. In camel milk similar to the human milk, α-lactalbumin (α-La) is the main protein of whey fraction; whereas, β-lactoglobulin (β-Lg) is absent. In contrast, in bovine milk, as a base of commercial infant milk powder, β-Lg is the main protein of whey fraction, which is considered as a potential source of food allergy in newborns. We have shown that bioactive peptides derived from camel milk proteins could have promising therapeutic properties such as anticancer, antioxidant, anti-hypertensive, antimicrobial and mineral binding effects in human body (Khalesi *et al,* 2017; Salami *et al,* 2008). Bioactive peptides are usually inactive in the native proteins and can be produced *in vivo* and *in vitro* by different digestive and microbial enzymes and by fermentation (Moslehishad *et al,* 2013. Angiotensin converting enzyme (ACE) inhibitory peptide is one of the bioactive peptides resulted from both enzymatic digestion and fermentation that can act actively to control blood pressure (Salami *et al,* 2011). Hydrolysis of camel and bovine milk whey proteins has shown that camel milk had more antioxidant and antimicrobial properties (Rahimi *et al,* 2016 and Salami *et al,* 2010). The anti-glycation of human serum albumin (HSA) and antioxidant effect of aloin, a major component of *Aloe vera*, in the presence and absence of casein-derived peptides from camel milk was studied (Moosavi-Movahedi *et al,* 2015). The presence of aloin and peptides reduced the number of glucose-attached lysine residues. Accordingly, aloin and camel casein derived peptides showed a synergic anti-glycation effect and inhibited

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the formation of fibrils during the HSA glycation. This effect can be related to the antioxidant activity of aloin–peptide complex (Moosavi-Movahedi *et al,* 2015). Curcumin is a protective component with anti-fibrils, antioxidant and anti-inflammatory properties. It is a potential component against various malignant diseases, diabetes, allergies, arthritis, Alzheimer's disease, cancer and other chronic illness (Mazaheri *et al,* 2015a and Mazaheri *et al,* 2015b). Curcumin is a natural polyphenol but poorly soluble in aqueous solutions. Fortunately, camel beta casein micelles are capable of increasing curcumin solubility up to 2500 folds (Esmaili *et al,* 2011). Accordingly, curcumin can be solubilised by camel beta casein for therapeutic purposes in human medicine.

A novel artificial enzyme was produced using camel β-casein (Cβ-casein). Peroxidase-like artificial enzyme, named "caseoperoxidase", was biomimetically designed using a nano artificial amino acid apo-protein hydrophobic pocket. This four-component nano artificial enzyme containing heme–imidazole–β-camel casein–SDS exhibited high activity growth and kinetics performance toward the native horseradish peroxidase. Cβ-casein was selected as an appropriate apo-protein for the heme active site because of its innate flexibility and exalted hydrophobicity. Camel β-casein as a hydrophobic protein has very suitable hydrophobic pocket that could accommodate the haeme prosthetic group inside its cavity (Moosavi-Movahedi *et al,* 2015).

In conclusion, camel milk can be revisited as superfood food with respect to protein content, bioactive peptides and casein micelles activities.

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MOLECULAR CLONING AND TISSUE EXPRESSION PATTERN OF PARTIAL HEPATIC GROWTH FACTOR cDNA IN ARABIAN CAMEL (*Camelus dromedarius*)

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ABSTRACT

The present study was performed to clone hepatic growth factor (HGF) in Arabian camel. RT-PCR was conducted on RNA from skeletal muscle using primers designed from the conserved regions in different animal species. The resultant PCR amplicon was subjected to sequencing and bioinformatics analysis. The results revealed that the obtained sequence belongs to HGF gene family. The nucleotides sequence was deposited in the GenBank with accession number KU736793. Furthermore, the data showed base frequencies of A = 32.77%, C = 21.67%, G = 22.06% and T = 23.5%. The nucleotide sequence alignment revealed that *Camels dromedarius* HGF showed 99% identity with *C. bactrianus* and *C. ferus* HGF, while it showed 97% identity with those of either *Bos taurus*, *Capra hircus*, *Ovis aries* and *Equus caballus*. Of the 766 nucleotides analysed, 36 substitutions varied from transitions and transversion were detected. The translated amino acids showed 2 non-synonymous substitutions discriminating camelids from other species; serine (S_{46}) into alanine (A_{46}), alanine (A_{46}) in other species at $T_{136}\rightarrow G_{136}$. The results showed a clear expression of HGF mRNA in a wide variety of the tested tissues; skeletal muscle, spleen, testes, liver, kidney and heart. The obtained results could be useful for more understanding of the structural-function relationship of HGF in Arabian camel and addressing the genetic diversity of the Arabian camel.

Key words: *Camelus dromedarius*, cloning, expression pattern, HGF

Camel has been historically and economically an important species worldwide and especially in Arab Peninsula. Saudi camels comprise 16% of the animal biomass (Al-Swailem *et al,* 2010). Although, dromedary camel has a high economic value in Saudi Arabia, the literatures about its reproduction biology and the molecular studies targeting its genome are limited. It is important to know the nucleotide sequence of various genes as this enables us to correlate between gene sequence and its functions to improve diagnosis of disease and improve drug design to target specific gene products that cause disease.

Many attempts have been carried out to identify some genes for single hump camel, especially in the Arabic regions including putative stress-induced heat-shock protein (Elrobh *et al,* 2011), putative copper-zinc SOD (Ataya *et al,* 2014) and c-Met (El-shazly *et al,* 2016). Growth factors are body proteins act to regulate cell division, differentiation and survival. They can also be produced through recombinant DNA technologies in the laboratory and used in the biological therapeutic purposes. HGF and its c-Met receptor are considered to be of the most important growth factors. HGF has molecular weight of 84 kDa (Nakamura *et al,* 1987; Nakamura and Mizuno, 2010). HGF is a dimeric molecule composed of an α-subunit (69 kDa) and a β-subunit (34 kDa), respectively linked by a disulfide bond. It gives two bands of 69 kDa and 34 kDa under reducing conditions. Thus, it is a heat-labile protein originally was discovered as a mitogen of adult rat hepatocytes (Nakamura *et al,* 1987; Nakamura and Mizuno, 2010). HGF is a multifunctional cytokine derived from stroma. It induces cell proliferation, differentiation and motility in a variety of epithelial cells by binding to the product of the c-Met proto-oncogene (Trusolino and Comoglio, 2002; Birchmeier *et al,* 2003; Yamaji *et al,* 2006). Moreover, HGF and c-Met have been involved in the embryonic and postnatal development of a variety of tissues including those of the mammary gland (Trusolino and Comoglio, 2002; Birchmeier *et al,* 2003; Yamaji *et al,* 2006). Whereas, HGF has a cytotoxic effect on certain tumour cells, such as haematoma, (HepG2.16) with regard to this, Higashio *et al* (1990) found that human lung fibroblasts (IMR-90) secrete a soluble factor named tumour cytotoxic

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factor (TCF) capable of killing Sarcoma-180. This group isolated TCF cDNA from the cDNA library of IMR-90 cells. As a consequence, TCF was found to be molecularly identical to HGF (Nakamura and Mizuno, 2010; Higashio *et al,* 1990).

The present study was aimed to clone hepatic growth factor (HGF) gene which is a key player in cell growth, differentiation and survival in addition to study its tissue distribution in Arabian camels.

Materials and Methods

Sampling

Different tissue samples were collected from the local slaughterhouse (Taif, Saudi Arabia). These tissue samples included samples from liver, kidney, spleen, heart, skeletal muscle and testes. Samples were exposed to sudden freezing in liquid nitrogen, transferred into laboratory and kept at - 80°C until used.

Primer Design

Two sets of degenerated primers were designed. The first (HGF-1F and HGF-1R) was from the highly conserved regions of HGF gene available in the gene bank for different species including *C. bactrianus* XM_010946631.1, *C. ferus* XM_006194335.2, Equidae (*Equus caballus* XM_014739144.1) and Bovidae (*Bos taurus* NM_001031751.2 and *Capra hircus* XM_018047278.1). The second one (HGF-2F and HGF-2R) was internal primers and was designed according to the obtained sequence and used for studying tissue distribution. Another set of primers (GAPDH-F and GAPDH-R) was for GAPDH and designed from the sequence of *C. dromedarius* GAPDH XM_010975572 (Table 1).

RNA Extraction, cDNA Synthesis and Reverse Transcription PCR

Total RNA was extracted according to the method described by Ahmed *et al* (2014) using Qiazol lysis reagent as per the manufacturer's instructions. Briefly, 100 mg of each tissue sample was homogenised in 1ml QIAzol (QIAGEN Inc., Valencia, CA) then 0.3 ml chloroform was added to the homogenate. The mixtures were shaken for 30 s followed by centrifugation at 4°C and 12,500 rpm for 20 min. The supernatant layer were transferred into a new set of tubes and an equal volumes of isopropanol were added to the samples, shaken for 15 seconds and centrifuged at 4°C and 12500 rpm for 15 min. The RNA pellets were washed with 70% ethanol, briefly dried up then, were dissolved in diethylpyrocarbonate (DEPC) water. The prepared RNA integrity was checked by electrophoresis. RNA concentration and purity were determined spectrophotometrically at 260 nm and 280 nm.

For synthesis of cDNA, mixture of 2 µg total RNA and 0.5 ng oligo dT primer in a total volume of 11 µl sterilised DEPC- water were incubated in the PeX 0.5 thermal cycler (Thermo Electronic Corporation, Milford, Ma) at 70°C for 10 min for denaturing. Then, 4 µl of 5X RT-buffer, 2 µl of 10 mM dNTPs and 100 U RevetAid Premium reverse transcriptase (Fermentas Canada Inc. Harrington Court, Burlington Ontario) were added and the total volume was completed up to 20 µl by DEPC water. The mixture was then re-incubated in the thermal cycler at 30°C for 10 min, at 42°C for 1 h and at 90°C for 10 min. The resulted cDNAwas preserved at -20°C until used.

Polymerase Chain Reaction ((PCR)

To amplify cDNA of HGF and GAPDH, polymerase chain reaction (PCR) and specific primers for each gene (Table 1) were used. PCR was conducted in a final volume of 50 µl consisting of 2 µl cDNA, 1µl (10 picomoles) of each primer and 25 µl PCR master mix (Promega Corporation, Madison, WI, USA) the volume was brought up to 50 µl using sterilised deionised water. PCR was carried out using a PeX 0.5 thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA) with the cycle sequence at 94°C for 5 min one cycle, followed by 35 (for HGF) and 25 (for GAPDH) cycles each of which consisted of denaturation at 94°C for 1 min, annealing at the specific temperature corresponding to each primer set (Table 1) and extension at 72°C for 1 min with an additional final extension at 72°C for 5 min. PCR products were electrophoresed on 1.5% agarose gel

Table 1. Primers and PCR conditions used for the tested dromedary genes.

Gene	Primer name and sequence (5` - 3`)	Annealing and Cycles	Product size
HGF	HGF1-F:AAAAGAAGAAACACACTTCATGAATTC HGF1-R: GCATTCAGTTGTTTCCATAGGG	54°C, 35 cycles	818 bP
HGF HGF	HGF2-F: CATTCCTCAGTGTTCAGAAG HGF2-R: TTGCCATTCCCACGATAACA	53°C, 35 cycles	454 bP
GAPDH (XM_010990867)	GAPDHF- TGGGAAGCTAACTGGCATG GAPDHR- AGGCAGGGCTCCCTAAGC	53°C, 25 cycles	550 bp

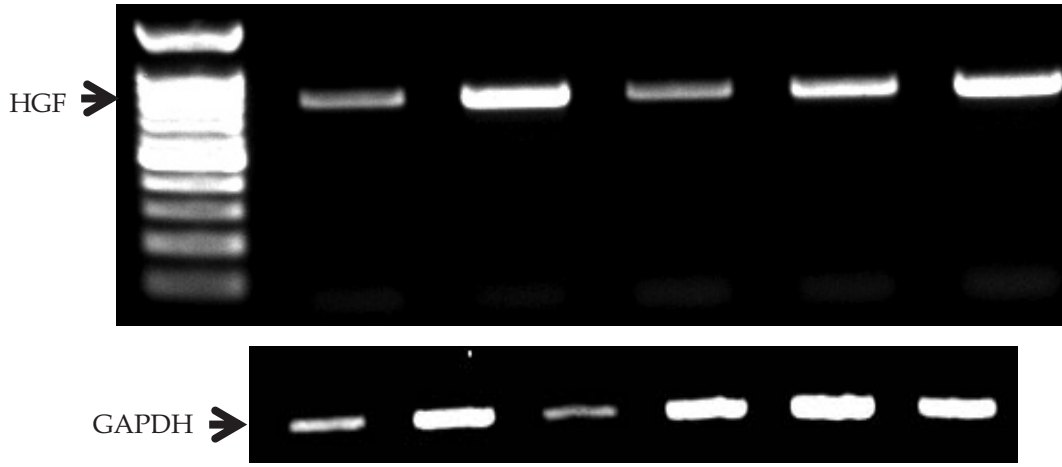

Fig 1. Expression of HGF in skeletal muscle from 5 different Arabian camels.

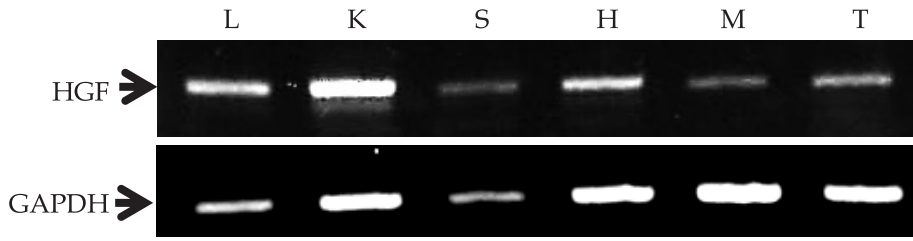

Fig 2. Tissue distribution of HGF mRNA Arabian camels. L: Liver, K: Kidney, S: Spleen, H: Heart, M: Muscle, T: Testis.

(Bio Basic, Konrad Cres, Markham, ON, Canada), stained with ethidium bromide in TAE (Tris–acetate–EDTA) buffer (Sigma–Aldrich, St. Louis, MO, USA). PCR products were visualised under UV light and photographed using gel documentation system (UVP, Upland, CA, USA). Following that, PCR product was purified using FavorPrep PCR Clean-Up mini kit according to the manufacturer's instructions.

Sequence analysis of PCR product

Purified PCR products for HGF were sequenced in an ABI PRISM 3730xl sequencer (Applied BioSystems) and BigDyeTM Terminator Sequencing Kits with AmpliTaq-DNA polymerase (FS enzyme) (Applied Biosystems) following the protocols supplied by the manufacturer. After reading the targeted genes, the nucleotide sequences have been treated with DNASIS software programs. Amino acid sequence was obtained by translating the sequenced DNA fragment using the DNASIS program and the deduced amino acid sequence was compared with sequences obtained from searches in the NCBI protein database using the BLASTP algorithm.

Results and Discussion

Several studies were conducted to identify some of the Arabian camel genome. Of these studies is Heat-Shock protein (Elrobh *et al,* 2011), putative Copper-Zinc SOD (Ataya *et al,* 2012), putative cytochrome P450s (Saeed *et al,* 2014), heat shock protein 90α (Saeed *et al,* 2015) or even mitochondrial genes (Ahmed *et al,* 2013) and Kappa casein (Minoia *et al,* 1998). The majority of studies performed on camel researches addressed mainly the milk and meat composition and how can it adapt to the harsh, arid climate which could be due to the unique physio-anatomical features of the Arabian camels (Saeed *et al,* 2015). The study of camel genome may give us a clear perception of the mechanism by which camel can adapt himself to hard climate conditions and resist diseases. Moreover, this may be helpful in molecular diagnosis of diseases and drugs designing.

Hepatic growth factor HGF is considered as a multifunctional cytokine with a receptor known as c-Met. They play an important role in the both embryonic and postnatal organ development (Yamaji *et al,* 2006). We have recently cloned the Arabian camel c-Met and studied its tissue expression pattern (El-Shazly *et al,* 2016). When HGF binds to its c-Met receptor, intracellular tyrosine kinase domain of c-Met β-chain undergoes autophosphorylation resulting in a wide range of biological effects including mitogenic, morphogenic and motogenic effects in different types

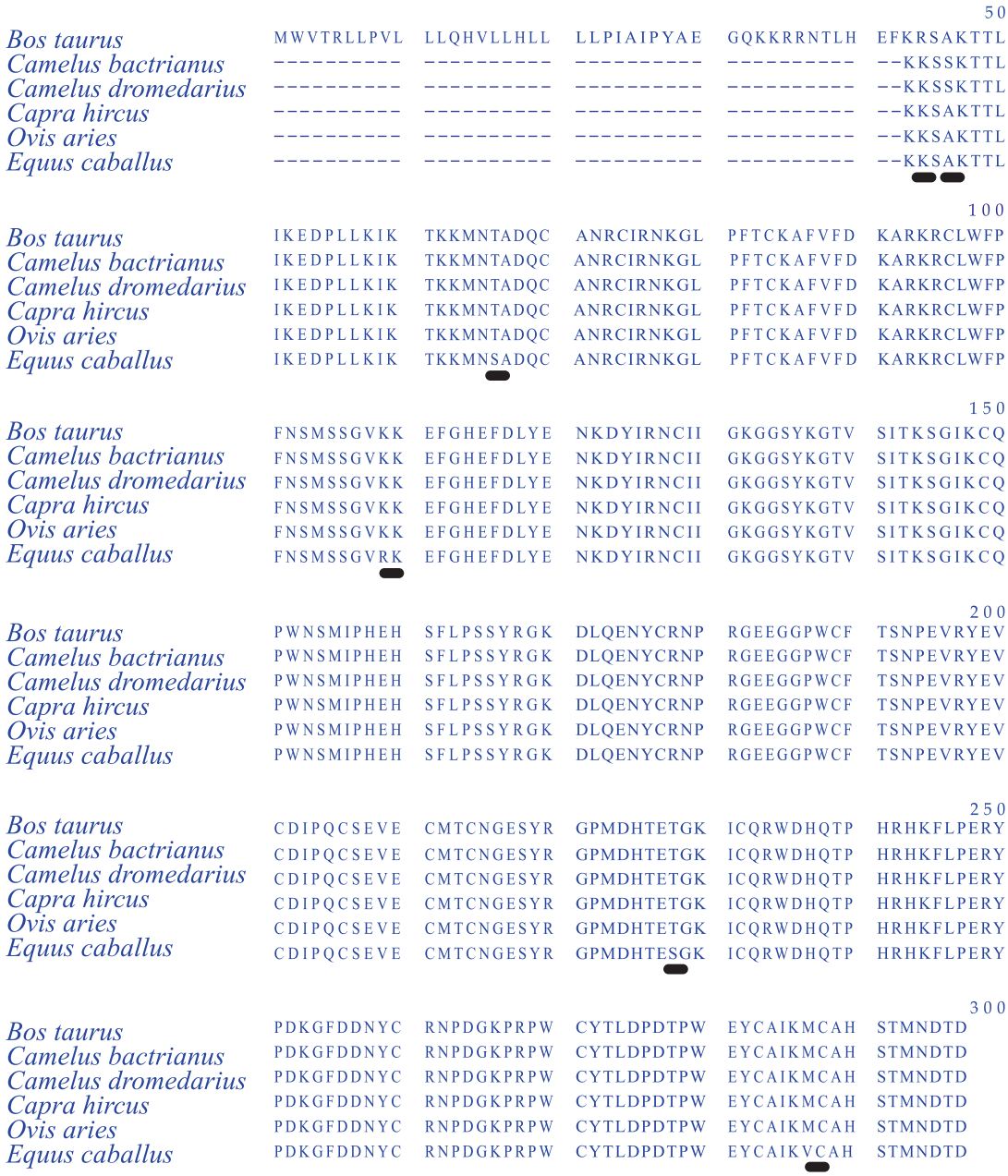

Fig 3. Amino acids alignment in different species.

of cells (Bottaro *et al,* 1991; Nakamura and Mizuno, 2010). Using cell-free cloning strategy, HGF gene of Arabian camel was partially cloned through PCR amplification technique. Primers HGF-1F and HGF-1R were used and a fragment of 818 bp was resulted (Fig 1). The PCR product was sequenced and a clear 766 bp peaks were analysed. The obtained nucleotide sequence (766 bp) of partial camel HGF cDNA was deposited in the GenBank data base under accession number of KU736793.

To analyse the obtained nucleotides sequences data, 766 nucleotides from Arabian camel HGF gene were aligned with their counterparts from in the Genbank database for the previously mentioned species. The data showed base frequencies of A = 32.77%, C = 21.67%, G = 22.06% and T = 23.5%. The nucleotide sequence alignment revealed that *C. dromedarius* HGF showed 99% identity with *Camelus bactrianus* and *C. ferus* HGF while it showed 97% identity with those of either *Bos taurus*, *Capra hircus*, *Ovis aries* or *Equus caballus* (Table 2). In addition, to 766 nucleotides analysed, 36 substitutions varied from transitions and transversion were detected. Of these 36 substitutions, only 6 were non-synonymous

resulted in amino acids substitution while the remaining 30 SNP were synonymous resulted in no amino acids substitutions among the aligned species (Fig 3). Among these substitutions, the translated amino acids showed one non-synonymous substitution at position 46 (Fig 3) discriminating the camelids from other species. These substation included change of serine (S_{46}) in the genus *Camelus* into alanine (A_{46}) in other species at $T_{136}\rightarrow G_{136}$. In addition, there were also two substitutions discriminating the non-ruminant, *Equus caballus* from the ruminants (*Bos taurus*, *Capra hircus*, *Ovis aries*) and pseudo-ruminants (*Camelus bactrianus*, *C. dromedarius*). That is arginine (R_{109}) at position 109 in *Equus caballus* was changed into lysine (K109) in the other species as a result of substitution of $G_{326}\rightarrow A_{326}$ and valine (V_{287}) at position 287 in *Equus caballus* was changed into methionine (M_{287}) in the other species as a result of substitution of $G_{859}\rightarrow A_{859}$. On the other hand, camelids (pseudo-ruminant) shared the non-ruminant (*Equus caballus*) in amino acids at positions 197, serine (S_{66}); serine (S_{228}) which was substituted by threonine (T_{197}) and (T_{228}) in other species (ruminants) as a result of change of $G_{197}\rightarrow C_{197}$ and $T_{682}\rightarrow A_{682}$, respectively (Fig 3). The numbers below the letters referred to the corresponding positions of either the amino acid or the nucleotide inside the complete gene sequence. To investigate the mRNA expression pattern of HGF tissue distribution, RT-PCR analysis using internal primers was conducted on different tissue samples. The obtained results revealed a wide range of HGF expression in different tissue including kidney, spleen, heart, liver testis and muscle.

Table 2. Identity percentage of *Camelus dromedarius* HGF with other species.

Animal species	HGF	
	Accession number	Identity
Camelus bactrianus	XM_010946631.1	99%
Camelus ferus	XM_006194335.2	99%
Bos taurus	AB110822.1	97%
Ovis aries	XM_012176561.2	97%
Equus caballus	XM_014739144.1	97%
Capra hircus	XM_018047278.1	97%

In conclusion, in the per cent study, we succeeded to partially clone HGF cDNA from Arabian camel tissue and investigate the mRNA expression pattern in some different tissues. Further studies are required to clone full length HGF gene from dromedaries which could be useful for more understanding of the structural-function relationship of HGF in Arabian camel.

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IDENTIFICATION AND MOLECULAR CLONING OF CYSTEINE PROTEASE GENE OF *Trypanosoma evansi* ISOLATED FROM CAMEL

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ABSTRACT

A molecular study was carried out to isolate cysteine protease gene of *Trypanosoma evansi* using PCR. The desired amplicons of cysteine protease gene from the genomic DNA of *T. evansi* were successfully amplified by PCR using gene specific primers at annealing temperature of 55°C. Amplified PCR product was identified on the basis of its size in agarose gel electrophoresis as 1533 bp. For cloning the purified DNA fragment was ligated to the pGEM- T Easy vector and the ligated mixture was transformed into *Escherichia coli* JM109 strains. The cells containing recombinant plasmid were identified on the basis of white/blue colony selection on LB agar containing X-Gal, IPTG and ampicillin. Screening of recombinants was done by restriction enzyme digestion of plasmid DNA using EcoRI and confirmed on the basis of gene size, i. e. 1533 bp for cysteine protease gene. Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies in the presence of insert specific primers.

Key words: Camel, cloning, cysteine protease, *Trypanosoma evansi*

Trypanosoma evansi is widely distributed amongst the domesticated and wild animals and it is the causative agent of trypanosomosis in camels, horses, mules, the Indian elephant, ruminants, etc (Gill, 1977). Trypanosomosis is the most pathogenic parasitic disease of camelids in all camel rising countries causing high morbidity and mortality (Luckins, 1992).

Proteases, also called peptidases are peptide-hydrolysing enzymes. Proteases are classified according to the initial position at which they cleave the peptide substrate. Based on reactive residues found in the active site, these enzymes are further categorised into 7 main groups: serine, cysteine, aspartic, metallo, threonine, glutamate and asparagine proteases (Rawlings *et al,* 2004). Cysteine proteases are good targets for comparative studies of trypanosomes because they serve vital role in development of trypanosomatids in their life cycles in vertebrate hosts and vectors contributing not only to the infectivity and pathogenesis but also to protective immune response (Atkinson *et al,* 2009; Caffrey and Steverding, 2009). Cysteine proteases have been characterised extensively in *T. cruzi*, *T. congolense* and *T. b. brucei* at the biochemical, molecular and immunological levels (Sajid and McKerrow, 2009; Lalmanach *et al,* 2002). Characterisation of the genes encoding cysteine proteases is helpful as first step in understanding cysteine protease enzymes in *T. evansi*. Thus, present study was carried out to isolate the cysteine protease gene of *Trypanosoma evansi* using PCR and cloning of the gene.

Materials and Methods

After confirmation of *T. evansi* infection by blood smear examination, blood from infected host was collected and inoculated intra-peritoneally in Swiss albino mice (maintained at Small Animal Laboratory, NRC on Camel, Bikaner). DNA isolation from collected pellet of *Trypanosoma evansi* was done as per the protocols given by ready to use kit from Illustra blood genomic prep. mini kit. The cysteine protease gene of *T. evansi* was amplified from genomic DNA using specific forward 5′ ACACCCACGCAAGCAGTAA 3′ and reverse 5′ ACGTCGATCGCGGACACATA 3′ primer sequences designed from published sequence of *T. cruzi* (Accession No. U41454.1). Cycling conditions for PCR were initial denaturation at 94°C for 4 minute, 35 cycles of 30 seconds at 94°C, 45 seconds at annealing temperatures of 55°C and 1 min. and 30 seconds at 72°C, followed by a final extension for 10 minute at

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72°C. The PCR amplified products were checked with 1.5 kb DNA molecular weight marker in 1% agarose gel.

The PCR product from low melting point agarose slices were purified using Illustra GFX PCR DNA and Gel Band Purification Kit. The DNA fragments of cysteine protease gene and the pGEM-T Easy vector in which it was to be cloned were digested with T4 DNA ligase enzyme to generate compatible ends for ligation. The ligation was done in the reaction volume of 20 µl containing 10µl of 2X Rapid ligation T4 DNA Ligase buffer [400mM Tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP (pH 7.8 at 25°C)], 6 µl PCR product, 2 µl pGEM- T Easy vector and 2 µl of T4 DNA ligase. The contents were mixed well by tapping and it was spun down in a micro centrifuge for 3-5 seconds and incubated for overnight at 4°C. The ligation mix was used directly for transformation in JM109 high efficiency competent cells. After incubation 100 µl transformation culture was plated onto antibiotic agar plates in duplicate and incubated at 37°C for overnight (16-20 hr). Colonies harbouring recombinant plasmids were inoculated into LB broth and incubated at 37°C overnight with horizontal shaking. The plasmid DNA was extracted from culture using illustra plasmid prep mini spin kit. The positive clone was confirmed by Restriction Enzyme digestion of plasmid DNAs with EcoR1 and colony PCR of plasmid colonies.

Results and Discussion

The genomic DNA was analysed in 0.8% analytical agarose gel and was found to be intact without much shearing. Gene specific forward and reverse primers were used for amplification of cysteine protease gene and the amplicons were analysed by agarose gel electrophoresis. An intensely amplified DNA was seen in lanes B and C using genomic DNA (Fig 1). The size of the intense band was deduced from the standard curve drawn between the log molecular sizes of the marker bands against their respective mobility and found to be 1533 bp.

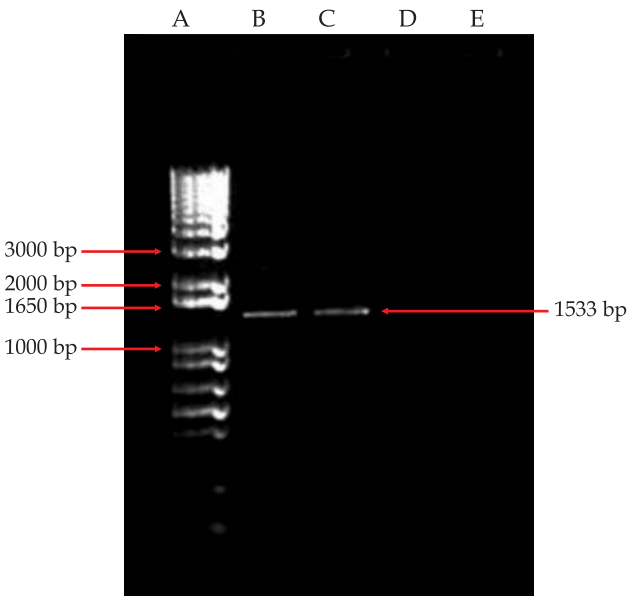

Fig 1. Amplification of cysteine protease gene of *T. evansi* by PCR. A. 1Kb plus DNA Ladder B-C. Amplicons.

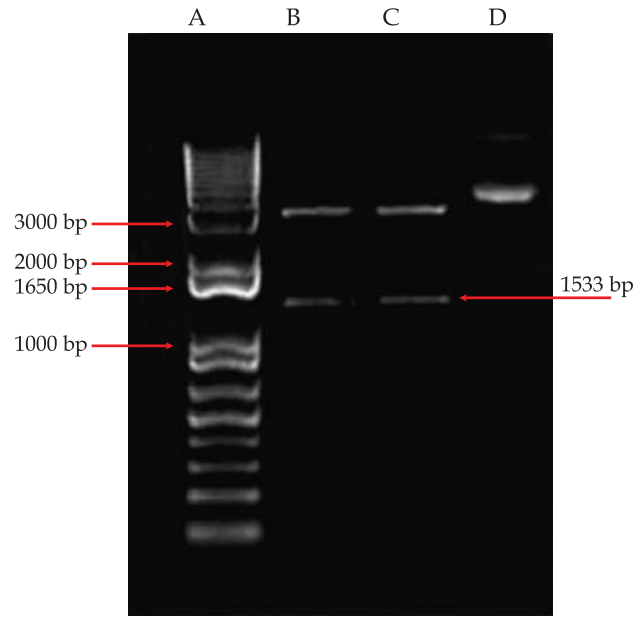

Fig 2. Cysteine Protease gene fragments of *T. evansi* after restriction digestion of *cysteine protease* plasmid Legends. A. 1Kb plus DNA Ladder B–C. *Cysteine protease* gene clone D. Uncut plasmid.

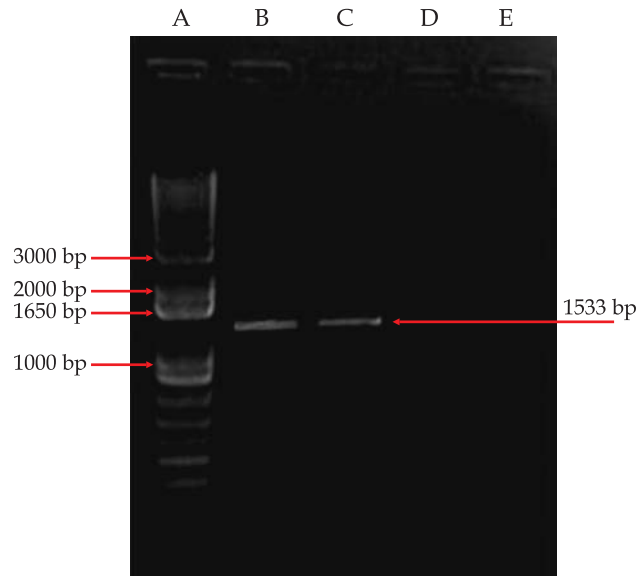

Fig 3. Amplification of Cysteine Protease gene of *T. evansi* by Colony- PCR. A. 1Kb plus DNA Ladder B–C. PCR reaction with white colony shows Amplification, D. PCR reaction with Blue colony shows no Amplification.

The amplified product was purified from the LMP agarose gel and ligated with pGEM-T Easy vector (Promega). 100 µl of transformation culture was plated onto X-gal-IPTG-Ampicillin agar plate and several white colonies along with a few blue colonies were obtained. The blue colonies represented the presence of vector alone but few of them contained vector with insert. The white colonies represent recombinant clones carrying insert in the plasmid. Two well separated DNA bands were seen in case of plasmid isolated from positive colonies upon digestion with Eco RI, the less intense lower band may correspond to the insert (Fig 2). Release of DNA fragments of around 1533 bp for cysteine protease gene was found after restriction enzyme digestion. Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies and amplification was found in wells of white colonies and also in blue colony (Fig 3).

The cysteine protease gene of *Trypanosoma evansi* is a nonvariant gene. Due to stability against the host immune response, this gene may be explored to make possible immune prophylaxis to control the disease effectively and inexpensively. It may also be investigated for identification of diagnostic markers and drug targets of this parasite to develop improved methods of prevention, diagnosis and treatment. In the present study, the amplicon size obtained was of 1533 bp. Gonzatti *et al* (1999) identified 453 bp partial cds of *Trypanosoma evansi* cysteine protease evansain gene (Accession no. AF165115). Tomas and Kelly (1996) reported *Trypanosoma cruzi* cysteine protease cruzipain gene of a tandem array complete cds of 1466 bp (Gen Bank Accession no. U41444) and Omara-Opyene and Gedamu (1998) observed that complete cds of the gene was 1677 bp (GenBank Accession no. AF004594). *Trypanosoma cruzi* cysteine protease gene, partial cds with 495 bp was identified by Eakin *et al* (1993; GenBank Accession no. M27305). However, in this study, the DNA fragment amplified in the PCR reaction was of expected size (1533bp) and highly target specific region of cysteine protease gene of *T. evansi*. With the cloning and sequencing of cysteine protease gene of *T. evansi* and expression of this protein it can make a great impact on the discovery of new protective antigen.

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EFFECT OF MIDAZOLAM SEDATION IN DROMEDARY CAMELS (*Camelus dromedarius*)

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ABSTRACT

The study was designed to evaluate the suitability of midazolam as sedative in dromedary camels. Midazolam was administered at a dose 0.4 mg kg-1 body weight, intravenously as a bolus in 6 camels for induction of sedation and determination of sedative effects on the behaviour and various physiological, haematological and biochemical parameters of camels were evaluated. Midazolam produced sedation of short duration (5-7 min) with mild to moderate depression of palpebral and corneal reflexes, moderate salivation and lacrimation, mild to moderate relaxation of jaw, tail, limbs, abdomen and anus. Rectal temperature and pulse rate showed non-significant increasing trend whereas, significant increase in respiration rate was observed at 5 and 10 min 12.83±0.83 and 12.66±0.42, respectively while significant decrease in respiration rate at 40 min 10.0±0.5 were observed, compared with base line value of 11.0 ± 0.36. No appreciable changes in haematological parameters and the activity of ALP, ALT, AST and LDH enzymes were observed in the present study. Intravenous administration of midazolam proved safe sedative agent when used alone for chemical restraint for a variety of diagnostic and minor surgical procedures of short duration in camels.

Key words: Camel, dromedary, midazolam, sedation

Many diagnostic and therapeutic procedures in camels can be accomplished with physical and/or chemical restraint techniques (Abrahamsen, 2009). In practice, deep sedation is commonly used in camel practice in field situations (Ismail, 2016). Prolonged surgical procedure or inadequate effects of general anaesthetic agent often require supplementation with local analgesic or general anaesthesia (White *et al,* 1987; Fahmy *et al,* 1995). The risk involved with the potentially life threatening drugs acting on the nervous system is due to lack of understanding of various physiological and pharmacological aspects in camels. Despite the great advances in the use and understanding of sedative drugs in domestic animals, there have been few reports of their use in camels (Fouad, 2000). Chloropromazine hydrochloride, propoinyl promazine and acepromazine have been evaluated as sedatives in camels (Khamis *et al,* 1973; Ali *et al,* 1989). Alpha-2 agonists like xylazine, detomidine, medetomidine and romifidine has also been used for restraint, the calming of camels or stress reduction (Ali, 1988). The sedatives used alone or in combination with general anaesthetics minimise the undesirable effects on the physiological status of the camels.

Midazolam is a water soluble imidazole benzodiazepine derivative with sedative, hypnotic, anticonvulsant and muscle relaxant properties (Marjorie, 2001), 4 times more potent than diazepam (Stegmann, 1998) thus, preferred over diazepam (Reves *et al,* 1985). Midazolam has an early induction, short duration, rapid elimination and total body clearance (Court and Greenblatt, 1992; Thurmon *et al,* 1996), has minimal effects on cardiopulmonary system (Lemke, 2007; Butola and Singh, 2007) and thus may be preferred for combination anaesthesia in camels. In veterinary practice, midazolam has been used as sedative/preanaesthetic in cattle calves (Bishnoi and Saini, 2005), alpacas (Aarnes *et al,* 2013), Pigs (Smith *et al,* 1991), buffaloes (Cheema, 2002; Malik, 2008) and in goats (Stegmann, 1998), have less side effects than diazepam (Conklin *et al,* 1980; Pieri, 1983). However, perusal of literature reveals little information on the use of midazolam in dromedary camels. Considering the importance of this species and the scarcity of information, the study was designed to evaluate the suitability of midazolam as sedative.

Materials and Methods

The present study was conducted on randomly selected 6 adult healthy camels of either sex, aged 9 - 13 years and weighing 350 - 600 kg body weight. The optimal sedative dose of midazolam was standardised by conducting pilot trials in camels administered

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intravenously. Out of various dose trials, the midazolam (0.4 mg kg^{-1} body wt, intravenously) resulted in complete sedation. The animals were restrained in sitting position; food and water were withheld for 24hrs prior to the experimental trial.

Experimental Procedure

Midazolam was administered at a dose 0.4 mg kg^{-1} body weight, intravenously as a bolus in 6 camels for induction of sedation and determination of sedative effects on the behaviour and various physiological, haematological and biochemical parameters was done. The blood samples (10 ml) were collected from the jugular vein in EDTA containing and plain glass tubes for haematological and biochemical analysis, respectively. The blood sample was centrifuged and the serum separated and stored in refrigerator until use.

Recording of Observations

The parameters investigated during study were recorded at base line, 5, 10, 20, 40, 60 minutes and after 24 hrs of intravenous administration of midazolam.

Clinico-physiological Observations

Depth of sedation/anaesthesia was assessed by monitoring various reflexes like palpebral and corneal reflex, position of eye ball, salivation, lacrimation, relaxation of jaw, limbs, tail, abdomen and anus and response to pin-prick and bone-prick. These observations were graded as mild (+), moderate (++) and good (+++). Relaxation of jaw, tail, limbs, abdomen and anus were graded according to resistance observed. Response to pin-prick and bone-prick was graded mild when animal blinked the eyes at the time of prick, moderate when animal shooks its skin and moved the tail, eyelids or nostrils and good when animal moved its limbs tail or head at the time of prick. Rectal temperature, pulse rate and respiration rate were also recorded.

Haemato-biochemical Parameters

Haemoglobin, packed cell volume, total erythrocyte count, total leucocyte count and differential leucocyte count were estimated. Biochemical study was performed for quantitative estimation of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactic dehydrogenase (LDH) enzymes on autoanalyser using standard kits.

The recorded data were subjected to the statistical analyses for interpretation of results to the methods suggested by Snedecor and Cochran (1967). The data were subjected to a two way analysis of variance (ANOVA) followed by a critical difference test for the comparison of mean values. A probability level of $P<0.05$ was considered as statistically significant. The mean values and standard error (SEM) were presented in tabular form.

Results and Discussion

Clinico-physiological Observations

Midazolam (0.4 mg kg^{-1}) administered intravenously as a sole agent produced change in normal behaviour of the camel (Table 1). Sanchez *et al* (1994) found midazolam (0.5 mg kg^{-1}) effective for restraint in cows. However, good sedation was observed in goats administered midazolam (0.4 mg kg^{-1}) intravenously (Stegmann, 1998). Midazolam (0.1–0.5 mg/kg) can cause paradoxic excitation, especially if administered rapidly intravenously to healthy adult ruminants (Seddighi and Doherty, 2016). The camels remained in sternal recumbency with the neck placed on the ground during onset of sedation however, lateral recumbency was observed in one camel at 10 min after intravenous administration of midazolam. It resulted in mild to moderate depression of palpebral and corneal reflexes, closure of eyelids with moderate salivation and lacrimation. The swallowing reflex decreased moderately during peak sedation. During sedation mild to moderate relaxation of jaw, tail, limbs, abdomen and anus was observed. Response to pin-prick was moderate at 5 min and mild to moderate at 10 min after administration. Response to bone - prick was found moderate at 5 and 10 min. The duration of sedation lasted for 5 - 7 min as shown by no response to pin-prick and bone-prick and absence of limb movements. Smith *et al* (1991) also observed a sedation of about 20 min. after single intramuscular injection or after incremental i.v. doses of midazolam in pigs. In another study, midazolam administered at a dose 0.5 mg kg^{-1} body wt. intravenously in buffalo calves induced sedation for 19.6 ± 2.61 min (Bishnoi, 2001). When used alone, midazolam could induce mild sedation for nonpainful procedures (e.g. radiography, ultrasonography) in tranquilised animals (Seddighi and Doherty, 2016). Midazolam causes muscle relaxation and mild tranquilisation, which when combined with anaesthetics like ketamine or propofol may improve the quality of induction or reduce the dose required to induce anesthesia (Pereira *et al,* 2006). Complete recovery from sedative effects occurred 35-40 min. after administration of midazolam evident as resumption of feed intake and walking without ataxia.

Table 1. Behavioural observations after intravenous administration of midazolam in camels (n = 6).

Observations	Time Interval (min)						
	Base Line	5	10	20	40	60	Hrs 24
Recumbency	**Sitting**	**Sitting**	**Lateral**	**Sitting/(Lateral)**	**Sitting**	**Sitting**	**-**
Palpebral Reflex	+++	++/(+++)	+/(++)	+++	+++	+++	+++
Corneal Reflex	+++	+++/(++)	++/(+++)	+++/(++)	+++	+++	+++
Eyeball Position	C	C	C/(D)	C/(D)	C	C	C
Salivation	-	+/(-)	++/(+)	+/++, (+/-)	-	-	-
Lacrimation	-	+/(++)	++/(+++)	+/++, (-)	-	-	-
Swallowing Reflex	+++	++	++	+++	+++	+++	+++
Jaw Relaxation	-	++	++	+	-	-	-
Tail Relaxation	-	++/ (+)	++	+	-	-	-
Limb Relaxation	-	+/(++)	++/(+)	-/(+)	-	-	-
Anus Relaxation	-	++	++/+++	+/(-)	-	-	-
Abdomen Relaxation	-	+/(-)	+/(-)	-	-	-	-
Pin Prick Response	+++	++	+/(++)	++/+++	+++	+++	+++
Bone Prick Response	+++	++/(+++)	++/(+)	+++	+++	+++	+++

- = Absent; + = Mild; ++ = Moderate; +++ = Good; C = Central; D = Downward rotation
Values in parentheses indicate variation from the response in one or two animals

The rectal temperature showed an increasing trend though statistically non-significant at different time intervals (Table 2). However, decreasing trend in rectal temperature was observed in buffalo calves till 45 minutes after administration of midazolam (Bishnoi, 2001) and at 60 minutes in pigs (Bustamante and Valverde, 1997) after administration of midazolam.

Pulse rate showed a non-significant increased value till 10 minutes while showed a regular decreasing trend at 20 min., till 24 hrs which are in accordance with the findings of Bishnoi (2001) in buffalo calves. However, significant increase in heart rate was observed in dogs (Jones *et al,* 1979). Non-significant change in cardio-pulmonary variables in goats was observed after administration of midazolam (Stegmann, 1998).

Significant increase in respiration rate was observed at 5 and 10 min 12.83 ± 0.83 and 12.66 ± 0.42, respectively, while significant decrease in respiration rate at 40 min.10.0 ± 0.5 were observed when compared with base line value of 11.0 ± 0.36. However, a significant decrease in respiration rate was seen in pigs (Smith *et al,* 1991) after midazolam administration. Bishnoi (2001) observed a non - significant decrease in the respiration rate in buffalo calves after administration of midazolam.

Haemato-biochemical Observations

No significant change was observed in the values of haemoglobin throughout experimental period. PCV value showed a non-significant increasing trend from 10 min. to 24 hrs while decreased at 5 min when compared to base line value. TEC, TLC and DLC did not show any appreciable change in their values in camels after administration of midazolam (Table 3). Bishnoi (2001) also reported non-significant change in haematological parameters in buffalo calves after administration of midazolam. Kashyap (1994) also reported non-significant change in hematological parameters in camels following administration of detomidine except for TLC values. However, a significant decrease in Hb and PCV along with decrease in TEC and TLC without any effect on DLC was observed in goats (Kumar and Thurmon, 1977) after intravenous administration of diazepam. The midazolam metabolites are conjugated and then excreted as glucuronides in the urine (Kronbach *et al,* 1989; Bauer *et al,* 1995). No appreciable changes in the activity of ALP, ALT, AST and LDH enzymes were observed in the present study (Table 4). Likewise, non-significant changes in activity of these enzymes had also been reported in buffalo calves after administration of midazolam (Bishnoi, 2001). In contrast to present study, an increase in activity of alkaline phosphatase after high doses of midazolam (45 mg kg^{-1}/day) was observed in dogs (Schlappi, 1983). Non-significant changes in the activity of ALP, ALT, AST and LDH enzymes were also observed in calves after diazepam sedation (Mirakhur *et al,* 1988). Kumar and Thurmon (1977) observed a mild increase in serum LDH and AST enzymes with no appreciable changes in ALP after diazepam administration in

Table 2. Physiological parameters after intravenous administration of midazolam in camels (n = 6).

Parameter	Time Interval (Min)						
	Base Line	5	10	20	40	60	24 Hrs
Rectal Temperature (° F)	97.51±0.35	97.69±0.31	98.08±0.31	98.19±0.27	98.43±0.23	98.71±0.21	97.90±0.26
Pulse Rate (Min^{-1})	43.16±0.56	46.33±0.95	48.16±0.57	47.00±2.95	45.83±2.89	44.83±2.37	–
Respiration Rate (Min^{-1})	11.00±0.36	12.83*±0.83	12.66*±0.42	11.00±0.73	10.00*±0. 51	10.86±0.80	10.66±0.73

* Significantly different from Base line value (P < 0.05).

Table 3. Haematological parameters after intravenous administration of midazolam in camels (n = 6).

Parameter	Time interval (min)						
	Base line	5	10	20	40	60	24 hrs
Hb (g/dl)	11.43±0.47	11.3±0.37	11.41±0.48	11.46±0.40	11.60±0.35	11.43±0.36	11.3±0.45
PCV (%)	27.16±0.87	26.16±0.94	27.33±1.02	27.66±0.84	27.33±0.95	28.5±0.99	27.6±0.98
TEC (x 10^6 μL^{-1})	9.33±0.49	9.16±0.40	9.66±0.33	10.16±0.16	10.00±0.00	9.33±0.33	9.33± 0.42
TLC (x 10^3 μL^{-1})	14.36±0.65	14.28±0.57	14.16±0.62	14.25±0.59	14.13±0.58	14.23± 0.66	14.26±0.66
DLC (%)							
Neutrophils	50.83±0.47	51.00± 0.68	51.16±0.47	51.33±0.33	50.83±0.60	50.66± 0.49	51.16± 0.40
Lymphocytes	41.00±1.06	40.83± 1.07	40.83±0.17	40.66±1.02	40.66±0.88	41.00± 1.06	41.0±0.93
Monocytes	2.0±0.25	2.0±0.25	2.00±0.25	1.83±0.30	1.5±0.22	1.50±0.22	2.0±0.25
Eosinophils	2.0±0.35	2.0±0.36	2.33±0.21	2.16±0.30	2.00±0.36	2.00±0.36	2.0±0.36

* Significantly different from Base line value (P < 0.05)

Table 4. Biochemical parameters after intravenous administration of midazolam in camels (n = 6).

Parameter	Time interval (min)						
	Base line	5	10	20	40	60	24 hrs
ALP (IU/L)	77.62±0.39	79.10±3.96	78.44±4.93	76.66±5.54	77.71±3.66	74.01±5.22	77.88±5.86
ALT (IU/L)	15.35±4.00	14.41±2.85	14.70±2.88	12.78±1.34	13.78 + 2.60	15.32 + 4.43	15.81±4.63
AST (IU/L)	118.11±21.18	125.25±22.76	124.29±22.05	123.12±21.06	118.31±5.87	112.98±6.08	120.53±21.14
LDH (IU/L)	555.81±46.34	567.40±50.02	564.59±48.02	560.75±53.65	542.01±49.11	596.56±44.31	571.59±50.44

* Significantly different from Base line value (P < 0.05)

goats. The non significant change in these enzymes favours its use in geriatric and compromised animals.

Thus intravenous administration of midazolam induces sedation for short duration at a dose (0.4 mg kg^{-1}). Midazolam could be used for chemical restraint for a variety of diagnostic and minor surgical procedures and declared safe sedative agent when used alone, or in combination with anaesthetic drugs to improve the quality of sedation in camels.

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EFFECT OF FEEDING DIFFERENT LEVELS OF ENERGY AND PROTEIN ON DRAUGHT PERFORMANCE AND PHYSIOLOGICAL PARAMETERS OF DROMEDARY CAMELS (*Camelus dromedarius*)

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ABSTRACT

The experiment was carried out to study the effect of feeding different levels of energy and protein along with groundnut haulms (*Arachis hypogaea*) on performance of dromedary camels. Three concentrate mixtures were formulated *viz*., high protein and low energy (T_1); high energy and low protein (T_2) and medium protein and energy (T_3). The digestible dry matter intake (DDMI) was 6.86, 6.61 and 7.5 kg/day, respectively in T_1, T_2 and T_3 which did not differ significantly from each other. The difference for DCP and TDN contents were significant between the treatment groups. There was significant difference for digestible crude protein intake (DCPI) and total digestible nutrient intake (TDNI) among the treatment groups. The power output and speed of operation was significantly ($P<0.05$) higher in T_3 as compared to others, but there was non-significant difference for draught (kgf). The camels in all the treatments were within the safe limit of physiological responses but there was significant ($P<0.05$) increase in the pulse and respiration rates after carting. The results of the study concluded that the performance of camels was higher in T_3 treatment as compared to either high protein or high energy supplementation.

Key words: Camels, draught performance, energy, protein

Camels are remarkable animals that have evolved with a ruminant like digestive system to enable them to survive on low quality, fibrous feeds. Being browsers, camels are able to select high quality diets, which they can efficiently digest. Camels have lower energy requirements than ruminants and have evolved an efficient mechanism for nutrient recycling. Camels have the ability to perform muscular functions such as racing at a level of intensity that exceeds the ability of horses. This unique capacity reflects the lower energy requirements for locomotion, the higher glucose supply, the lower oxygen demand and preferential dependence on slow twitch muscle fibres which in turn rely on aerobic metabolic pathways.

The one humped camel (*Camelus dromedarius*) are adapted themselves to the ecosystem of dry and arid zones where are subjected to harsh conditions in addition to the severe fluctuations in the nutritional status, which in turn affect their general performance (Nazik *et al*, 2015). Guidelines for camel feeding have often been extrapolated from the feeding standards for cattle, assuming that the digestibility of foods by camels and their efficiency of utilisation of nutrients for various functions do not differ significantly from those of true ruminants (Hashi and Kamoun, 1995). The present investigation was carried out to study the effect of feeding different levels of energy and protein on draught performance and physiological parameters of dromedary camels (*Camelus dromedarius*).

Materials and Methods

The experiment was conducted using 9 Bikaneri male camels of 8-9 years of age with an average body weight of 590 to 640 kg. Three concentrate mixtures were formulated *viz*., high protein and low energy (T_1); high energy and low protein (T_2) and medium protein and energy (T_3). The groundnut haulms (*Arachis hypogaea L.*) was offered free of choice to all the camels as basal roughage. Concentrate mixtures were formulated on the farm by using wheat bran (*Triticum aestivum*), groundnut cake (*Arachis hypogaea*), barley (*Hordeum vulgare*), moth meal (*Vigna aconitifolia*), salt and mineral mixture. While preparing concentrate mixtures, groundnut cake and barley were first ground in hammer mill and then all the ingredients were mixed evenly. Concentrate mixtures were prepared at monthly intervals using

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the ingredients from the same lot purchased at the start of experiment. Feeding was done twice daily i.e. in the morning as well as in the evening and feed refusal was weighed once daily prior to morning feeding. The amount of concentrate fed was calculated on the basis of estimated requirement of camels as per Indian Council for Agricultural Research (1985). During the metabolic trial, the representative samples of feeds and faeces were pooled and analysed for proximate principals (AOAC, 2000). The camels were housed in well ventilated shed having sandy floor, asbestos roofing and provision for manger for individual feeding. Before the start of the experiment, the animals were vaccinated, wormed and adapted to the feeds. The camels were weighed fortnightly after 16 hours fasting to reduce the gut-fill, thereby minimising the weight fluctuations.

The animals were trained for carting and had developed endurance for working for 4-6 hr daily. A 2 wheeled camel cart was used as a loading device and load cell of 500 kg capacity was used for measuring the draught. The cart was pulled on a sandy track to cover an approximate distance of 25.5 km daily in 4 to 5 hrs. The camels were allowed to pull payload including the weight of cart and the driver in such way that the experimental camels could exert an average draught of 18 per cent of their body weight. The speed and power developed by camels were calculated for all the experimental camels.

The draught was recorded during the experiment and power was calculated using the standard formula:

$$p = \frac{dxs}{270}$$

Where,

p= Power developed, hp

d= Draught, kgf

s= Average speed, kmh^{-1}

The physiological responses such as respiration rate (flank movement), pulse rate (coccygeal pulsation) and body temperature of camels were recorded before and after the draught stress. The experiment was conducted in completely randomised design and statistical analysis of the data was carried out by one-way ANOVA as suggested by Snedecor and Cochran (1994).

Results and Discussion

The overall crude protein (CP) and total digestible nutrient (TDN) content of concentrate mixtures offered were 23.27 and 65.65, 13.13 and 74.89, 16.49 and 70.71, respectively in T_1, T_2 and T_3. The groundnut haulms offered as basal roughage contained 9.01% CP, 1.78% EE, 24.73% CF, 14.82% TA, 41.51% NDF, 29.39% ADF and 12.12% hemicelluloses. The crude protein content (9.2%) was higher in the present investigation as compared to the reports of Gupta *et al* (2012) but lower than that reported by Bui (1998) for peanut haulms. However, the crude fibre (CF) and nitrogen free extract (NFE) contents were lower than that reported by Chaudhary *et al* (2008) who fed different levels of energy along with groundnut straw to draught camels.

The DCP content was significantly ($P<0.05$) higher in T_1 as compared to T_3 and T_2 which might be due to feeding of camels on higher levels of protein (Nagpal *et al*, 2011 and Gupta *et al*, 2012). The total digestible nutrient (TDN) was higher in T_3 which was of the order of 5.17 and 10.25 per cent units over that of T_2 and T_1, respectively which was supported by Chaudhary *et al* (2008) who reported similar trend for TDN content in the ration of dromedary camels. The DMI, DDMI and DOMI (kg/day) did not differ significantly ($P<0.05$) among treatment groups which may be due the fact that the type of feed and fodder did not affect the dry matter intake (Rai *et al*, 1994; Nagpal *et al*, 2010 and Gupta *et al*, 2012). The DCP intake was higher in T_1 as compared to T_3 and T_2 which might be due to feeding of camels with higher levels of protein in T_1 (Nagpal *et al*, 2011). The TDN intake (kg/day) was significantly ($P<0.05$) higher in T_3 (7.43) as compared to T_2 (6.86) and T_1 (6.24) which was in accordance with the findings of Gupta *et al* (2008).

The average daily gain (g/day) was not affected by the treatment groups. There was non-significant difference between the treatments for draught performance (kgf). The speed of operation

Table 1. Proportion of ingredients in concentrate mixtures (%).

Feed	Treatments		
	High Protein and Low Energy (T_1)	High Energy and Low Protein (T_2)	Medium Protein and Energy (T_3)
Triticum aestivum bran	33.33	13.89	28.57
Arachis hypogaea cake	33.33	8.33	14.28
Hordeum vulgare	16.67	69.44	42.86
Vigna aconitifolia meal	16.67	8.34	14.29
Crude Protein	23.27	13.13	16.49
Estimated TDN	65.67	74.89	70.71

Table 2. Proximate composition (% DM basis) of feed and fodder offered to draught camels.

Feed	DM	OM	CP	EE	CF	TA	NFE
Triticum aestivum bran	90.29	95.2	12.25	2.96	8.89	4.8	71.1
Arachis hypogaea cake	92.91	92.8	43.2	8.79	9.2	7.2	31.61
Hordeum vulgare	90.67	95.5	9.5	2.15	6.68	4.5	77.17
Vigna aconitifolia meal	91.52	89.8	19.25	5.97	10.67	10.2	53.91
Arachis hypogaea straw	90.84	85.18	9.01	1.78	24.73	14.82	49.66

Table 3. Nutrient utilisation in dromedary camels.

Attribute	Treatments		
	T_1	T_2	T_3
DMI, kg/day	10.95±0.70	10.89±0.88	11.69±0.10
DDMI, kg/day	6.86±0.64	6.61±0.77	7.51±0.18
DOMI, kg/day	10.95±0.77	10.89±0.88	11.69±0.10
DCPI, g/day	886.62±41.51^a	693.08±58.14^b	840.73±8.90^a
TDNI, kg/day	6.24±0.32^c	6.86±0.58^b	7.43±0.16^a
DCP, %	8.11±0.29^a	6.36±0.10^c	7.19±0.05^b
TDN, %	57.06±1.52^c	60.29±0.86^b	63.58±1.15^a

^{a,b,c} Mean values in the same row that have different superscripts are significantly different from each other (P<0.05).

Table 4. Body weight and draught performance in dromedary camels.

Attributes	Treatments		
	T_1	T_2	T_3
Body weight, kg			
Initial body weight	639.00±52.30	632.33±55.32	628.66±55.07
Final body weight	650.00±48.25	642.70±52.13	640.66±51.12
Body weight change	11.00±4.27	10.36±5.91	12.00±5.95
Average daily gain, g/day	183.33±71.20	172.78±98.63	199.99±99.28
Draught performance			
Draught, kgf	117.00±8.68	115.68±9.38	115.32±9.20
Speed, km/h	2.82±0.01^c	2.88±0.09^{bc}	3.29±0.01^a
Power, hp	1.22±0.09^c	1.23±0.14^{bc}	1.44±0.11^a

^{a,b,c} Mean values in the same row that have different superscripts are significantly different from each other (P<0.05).

was significantly (P<0.05) higher in T3 but there was non-significant difference between T_1 and T_2 for speed of operation (Gupta *et al*, 2014). Likewise, the power output (hp) was 1.44, 1.23 and 1.22 in T_1, T_2 and T_3, respectively which was significantly (<0.05) higher in

Table 5. Physiological parameters in dromedary camels

Attributes	Treatments		
	T_1	T_2	T_3
Rectal Temperature, °C			
Before work	36.73±0.68	36.43±0.75	36.93±0.40
After work	38.70±0.34	38.30±0.60	37.96±0.41
% Increase	5.35	5.12	2.80
Pulse rate, beats/minute			
Before work	45.66±0.57	46.33±1.52	45.66±0.58
After work	55.33±0.56^a	53.00±0.58^b	50.00±1.15^c
% Increase	21.17	14.39	9.49
Respiration rate, breaths/minute			
Before work	8.66±0.57	9.00±1.00	8.33±1.15
After work	18.66±0.58^a	17.00±1.01^a	12.66±1.16^b
% Increase	115.38	88.89	52.00

^{a,b,c} Mean values in the same row that have different superscripts are significantly different from each other (P<0.05).

T_3 as compared to T_1 and T_2. The results for draught performance in camels were within the range as reported by Rai and Khanna (1994) who reported the similar trend.

There was non-significant difference between the treatment groups for rectal temperature but there was increase in rectal temperature when camels were put under work. The pulse rate (beats/minute) was significantly higher in T_1 followed by T_2 and T_3. The per cent increase in pulse rate in camels before and after work was of the order of 21.17, 14.39 and 9.49, respectively in T_1, T_2 and T_3. There was significant increase in the respiration rate (breaths/minute) in camels before and after work which was confirmed by Khanna and Rai (2000) who reported increase in respiration rate after carting in draught camels. Similarly, Rai and Khanna (1994) reported an increase in body temperature, pulse rate and respiration rate over the initial values in Bikaneri camels. The increase in physiological parameters might be due to higher heat stress and hard muscle exercise during carting and lower availability of energy in the body.

Conclusions

The results concluded that the dromedary camels may be fed on ration having medium level of protein and energy rather than higher energy and lower protein or high protein and low energy through concentrate mixture along with leguminous based roughages for improved draught performance without showing fatigue symptoms.

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DETECTION OF GENETIC VARIATIONS AMONG *Staphylococcus aureus* ASSOCIATED WITH CAMEL SKIN WOUNDS ON THE BASIS OF *spa* AND *coa* GENE

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ABSTRACT

The present study was carried out to determine genetic variations among 26 isolates of *Staphylococcus aureus* obtained from camel skin wounds. The variations were detected by polymorphisms pattern of protein A (*spa*) and coagulase (*coa*) gene including RFLP of *coa* gene. Twenty five isolates produced 7 different *spa* types (amplicon size varied between 160 and 300 bp) while 1 isolate did not show amplification of the gene. Of the 25 *spa* gene positive isolates, 24 isolates produced only single amplicon whereas, one isolate produced 2 amplicons of 260 bp and 300 bp size. All the isolates produced single *coa* gene amplicon of variable size *viz*. 510 bp, 600 bp, 680 bp, 710 bp and 760 bp size. Six RFLP patterns were detected after digestion with *Alu*I restriction endonuclease enzyme. Among different RFLP patterns, PI and PIII were found genetically closer while PVI was most distant.

Key words: Camel, *coa* gene, genetic variability, *spa* gene, *Staphylococcus aureus*

Staphylococcus aureus is the most important pathogen causing abscesses, wounds and other skin lesions such as boils, styes and furuncles in camel (Qureshi *et al,* 2002; Rathore *et al,* 2012; Lakshmi, 2015). The organism shows variations in phenotypic properties, virulence and response to antibiotic treatment. Hence, there is need to study this organism in regard to genetic variations on the basis of virulence associated genes.

Protein A is an immune evasion protein secreted by *S. aureus* and is encoded by *spa* gene. It is considered as one of the important virulence factors in the development and severity of infection (Akineden *et al,* 2001). Several studies have reported the genetic diversity in *spa* gene and also related the number of nucleotide repeats to the pathogenicity of the organisms (Karahan *et al,* 2011; Rathore *et al,* 2012). Variations among *S. aureus* strains on the basis of *spa* gene come from the differences in the repetitive variable number of 24 bp repeats in x-region of the gene and this property is being used as a molecular tool in studying the genetic diversity among the strains of *S. aureus* for epidemiological tracing of source of infection and comparing the differences in virulent phenotypes (Bhati *et al,* 2016).

Coagulase is an extracellular protein established with significant role in virulence, encoded by *coa* gene that possesses polymorphic repeat region comprising of 81 bp tandem short sequence repeats (SSRs). Thus profiling of *coa* gene and its restriction fragment length polymorphism (RFLP) can be used to measure relatedness or variations among *S. aureus* isolates (Ishino *et al,* 2007; Coelho *et al,* 2009; Saei *et al,* 2009). The PCR-RFLP of *coa* gene is a rapid, simple and efficient method for typing strains, tracing the source and transmission route of *S. aureus* infection helping to prevent and control infections (Roodmajani *et al,* 2014). The characterisation of *S. aureus* on the basis of *spa* and *coa* gene can be considered a simple and accurate method to detect molecular variations of *S. aureus* isolates (Rathore *et al,* 2012; Bhati *et al,* 2014; 2016). In the preset study, *S. aureus* isolates obtained from camel skin wounds were subjected to polymerase chain reactions taking *spa* and *coa* as target genes to see variations among them.

Materials and Methods

Sample

Forty one samples were collected from camels out of which 26 genotypically confirmed isolates

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were included in present study. All the isolates were confirmed by 23S rRNA gene ribotyping (Straub *et al,* 1999).

Spa gene amplification

The *spa* gene amplification was carried out as per method described by Frenay *et al* (1996) with some modifications. The forward and reverse primers used were 5′-CAAGCACCAAAAGAGGAA-3′ and 5′-CACCAGGTTTAACGACAT-3′, respectively. The PCR mixture (25 µl), was prepared by mixing F-primer, 1.0 µl (75 pmol/µl), R-primer, 1.0 µl (75 pmol/µl), 5 µl 10× Taq buffer A containing 15 mM $MgCl_2$, 1 unit of Taq polymerase (5 U/µl), 0.5 µl dNTP mix (10 mM/µl), deionised water 11.8 µl and DNA template 3.0 µl (25 ng/µl). The PCR was performed in 35 cycles. The denaturation, primer annealing and primer extension was carried out at 94, 55 and at 70°C, respectively for 60 s for cycles 1 to 34. The final extension was carried out at 72°C for 5 min for cycle 35. The PCR products were resolved in 1.2 % agarose gels with 50 bp molecular marker as ladder (Fig 1).

Coa gene amplification

Amplification of *coa* gene was carried out as described by Hookey *et al* (1998) using primers with sequence of 5′-ATAGAGATGCTGGTACAGG-3′ (Forward) and 5′-GCTTCCGATTGTTCGATGC-3′ (Reverse). The PCR mixture (25 µl), was prepared by mixing forward primer, 1.0 µl (10 pmol/µl), reverse primer, 1.0 µl (10 pmol/µl), 5 µl 10× Taq buffer A containing 15 mM $Mgcl_2$, 1 unit of Taq DNA polymerase (5 U/µl), 0.5 µl dNTP mix (10 mM/µl), deionised water 11.8 µl and DNA template 3.0 µl (25 ng/µl). The PCR was performed in 30 cycles. The denaturation, primer annealing and primer extension was carried out at 94, 57 and at 70°C, respectively, for cycles 1–29 and at 94, 57 and 72°C, respectively, for cycle 30. The time given for denaturation, primer annealing and primer extension was 45, 15 and 15 s for cycle 1; 20, 15 and 15 s for cycles 2 to 29; and 20, 15 and 120 s for cycle 30, respectively. The PCR products were resolved in 1.2% agarose gels with 100 bp molecular marker as ladder (Fig 2).

Restriction fragment length polymorphism (RFLP) of coa gene

The RFLP of *coa* gene was carried out with *Alu*I restriction endonuclease enzyme as described by (Hookey *et al,* 1998). The PCR product (10 µl) was added with nuclease free water (5 µl), 10× Buffer Tango (2 µl) and *Alu*I (2 units, conc. of stock enzyme was 5 U/µl), was mixed gently and incubated at 37°C for 3h. The digests were resolved in 2% MetaPhor agarose gels with 50 bp and 100 bp molecular marker (Fig 3).

Cluster Analysis

To find the genetic relatedness, cluster analysis was carried out by Dice method and cluster as mentioned in Fig 4 formed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (http://insilico.ehu.es/dice_upgma/ online software support.

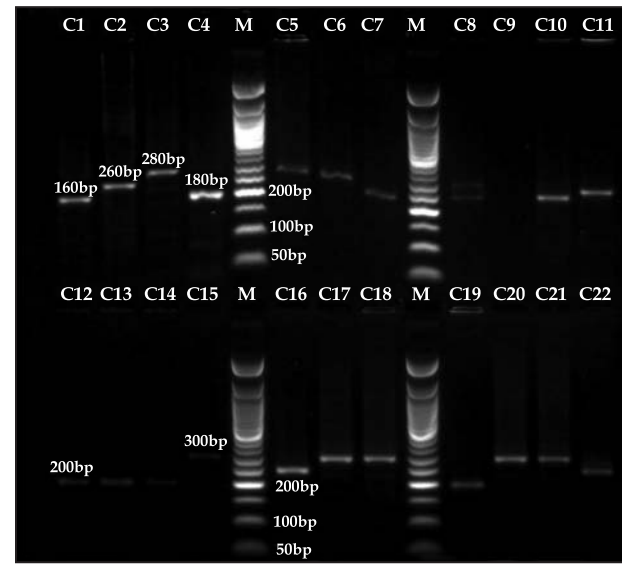

Fig 1. C19–Isolate showing 2 amplicon of *spa* gene.

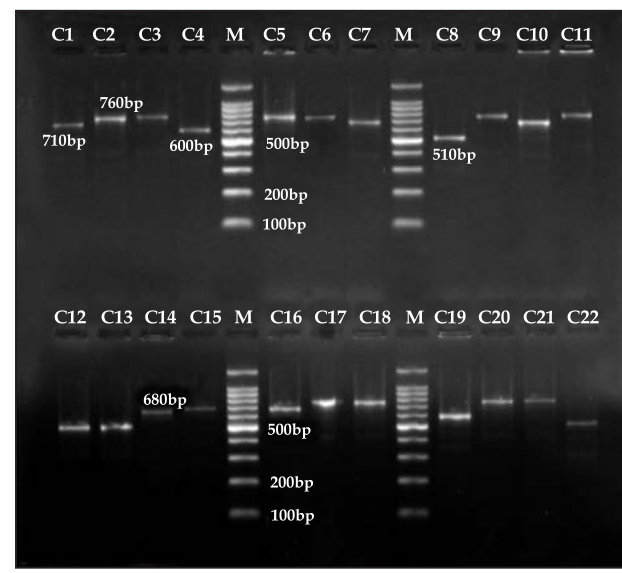

Fig 2. *Coa* gene amplicfication in *S. aureus* isolates from camel skin wounds C1 to C22, M–100 bp DNA ladder.

Table 1. *Spa* gene variations in *S. aureus* isolates from camel skin wound.

S.No.	Isolates number	Total isolates (%)	Size of *spa* amplicon (bp)	Total number of repeats
1.	C1	1 (3.84)	160	5
2.	C4	1 (38.46)	180	6
3.	C12, C13, C14, C19	4 (15.38)	200	6
4.	C2, C6, C7, C10, C16, C24	6 (23.07)	260	9
5.	C3, C5, C11, C20, C22	5 (19.23)	280	10
6.	C15, C17, C18, C21, C23, C25, C26	7 (26.92)	300	11
7.	C8	1(38.46%)	260, 300	9 and 11
8.	C9	1(38.46%)	Absent	–

Results and Discussion

The camel is comparatively less prone to many of the diseases, however, the skin infections including wounds and abscesses are a problem in camel resulting into its reduced working efficiency (Wernery *et al,* 2014). The most common organism in skin wounds has been found to be *S. aureus*. The present investigation was carried out with a view to find out genetic variations among *S. aureus* especially, in relation to *spa* and *coa* virulence genes. All the isolates in the study were confirmed by 23S rRNA based ribotyping which produced 1250 bp species specific amplicons.

Variations in spa gene

The x-region of *spa* gene was amplified in 25 isolates while one isolates (C9) did not produce any *spa* amplicon. The isolates were divisible into 7 *spa* types depending on the size as described in table 1. The amplicons obtained were 160 bp, 180 bp, 200 bp, 260 bp, 280 bp and 300 bp with calculated number of repeats of 5, 6, 6, 9, 10 and 11, respectively. All isolates produced a single amplicon except one (C8) which produced 2 amplicons. The amplicon of 300 bp was detected in 7 isolates followed by 260 bp amplicon in 6, 280 bp amplicon in 5 and 200 bp amplicon in 4 and remaining amplicons of 160 bp and 180 bp in 1 isolate each (Fig 1). The *spa* types obtained in the present study are similar to those reported by Rathore *et al* (2012) who observed amplicons of similar sizes in *S. aureus* isolates obtained from camel skin wounds and abscesses. Eventhough, they recorded presence of an additional 500 bp size amplicon but did not record any isolate without *spa* gene.

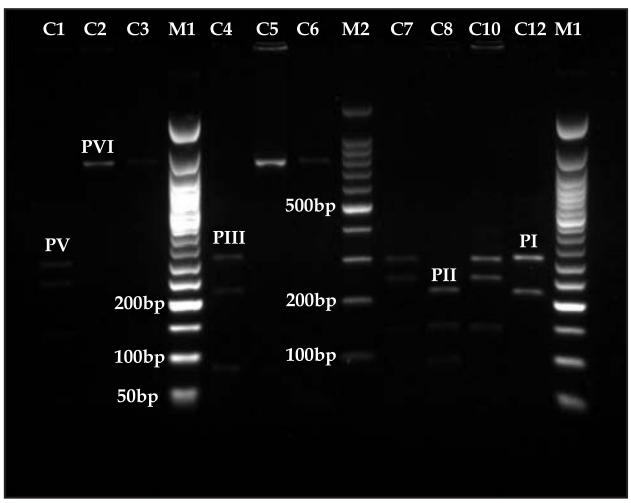

Fig 3. Different RFLP patterns of coa gene of *S. aureus* isolates C1 to C12, PI to PVI–Various RFLP patterns M1–50 bp DNA ladder, M2–100 bp DNA ladder.

The results in the present study are in conformity to those of Yadav *et al* (2015a) from the same area of study who reported that all their 32 *S. aureus* isolates obtained from milk of cattle (16) and buffalo (16) were divisible into 7 *spa* types with amplicon sizes ranging between 120 bp and 380 bp. The detection of 2 (260 and 300 bp) *spa* amplicons in one isolate in our study is in conformity to the observations of Shakeri *et al* (2010) and Bhati *et al* (2016). In the present investigation, one isolate did not produce *spa* gene amplicon which corroborated earlier observations of Kalorey *et al* (2007), Baum *et al* (2009), Momtaz *et al* (2010), Salem–Bekhit *et al* (2010), Khichar *et al* (2014) and Bhati *et al* (2016) who recorded non-*spa* typable strain of *S. aureus* and suggested that in these strains either *spa* mutation occurred or *spa* was absent. In the present study, 19 isolates were considered to be pathogenic since they possessed more than 7 repeats, as suggested by Frenay *et al* (1996). On the other hand, no correlation was reported between number of tandem repeats and pathogenicity of the isolates by other workers (Nashev *et al,* 2004; Kuzma *et al,* 2005; Jakubczak *et al,* 2007 and Kurlenda *et al,* 2010).

Variations in coa gene

All the 26 *S. aureus* isolates showed amplification of *coa* gene and they also produced free coagulase as detected in tube test. In our study, we obtained 5

coagulase types based on variable amplicon size where amplicons of 510 bp, 600 bp, 680 bp, 710 bp and 760 bp were produced by 4, 2, 4, 6 and 10 isolates, respectively (Fig 2; Table 2). The amplicon sizes obtained in the present study were much similar to that obtained by Salasia *et al* (2004) who carried out *coa* gene typing using the similar primers as in the present study, with *S. aureus* isolates from bovine sub-clinical mastitis. From the present study area Rathore *et al* (2012) reported 600 bp, 710 bp, 760 bp and 850 bp isolates from camels skin isolates, Khichar *et al* (2014) reported 510 bp, 600 bp, 710 bp and 850 bp amplicons from cattle mastitis isolates and Bhati *et al* (2014) reported 400 bp, 490 bp, 510 bp, 550 bp, 600 bp, 710 bp, 760 bp, 810 bp and 850 bp amplicons from cattle subclinical mastitis.

The above results indicated that of the 5 coagulase types in the present study three (600 bp, 710 bp and 760 bp) were similar to those reported by Rathore *et al* (2012) in camel wounds and abscesses isolates. The studies from the same area revealed that amplicon of 600 bp was produced by all the *S. aureus* isolates irrespective of type of infection and animal species from where the isolates were recovered. The amplicons obtained in the present study were also reported by one or more workers from the same area. A huge polymorphism in *coa* gene of *S. aureus* isolates from the area may be due to deletion or insertion mutations changing the *coa* gene size as suggested by El-Jakee *et al* (2010).

RFLP of coa gene

Restriction fragment length polymorphism has been found to be very useful tool in differentiating various strains based on digestion of amplicons with endonucleases resulting into fragments of variable lengths. Hookey *et al* (1998) were first to describe a coagulase based PCR RFLP technique that differentiated the major epidemic methicillin resistant *S. aureus* strains and minor epidemic strains. In the present investigation of the 5 coagulase types only 4 types (510 bp, 600 bp, 680 bp and 710 bp) were digested whereas 1 with 760 bp amplicon (from 10 isolates) remained undigested (Fig 3). The number of fragments produced upon *Alu*I digestion varied from 2 to 3 and their sizes varied from 80 bp to 300 bp. Overall six RFLP patterns (PI to PVI) were generated after digestion (Table 3). In our study, fragments of similar sizes were detected among different sized coagulase types *viz.* fragment of 80 bp was obtained with digests of 510 bp and 600 bp coagulase types, fragment of 150 bp was obtained with digests of 510 bp and 710 bp coagulase types, fragment of 210 bp was obtained with digests of 510 bp, 600 bp and 680 bp coagulase types, fragment of 260 bp size was obtained with digests of 680 bp and 710 bp coagulase types and fragment of 300 bp was obtained with digests of 510 bp, 600 bp and 710 bp coagulase types. The presence of similar fragments in different amplicons reveals common sites for endonucleases to act upon. On RFLP of *coa* gene similar fragments were also reported by some workers in mastitis isolates from the same area of study *viz.* Sanjiv *et al* (2008), Upadhyay *et al* (2012) and Bhati *et al* (2014) who reported fragments of 210 bp, 260 bp and 300 bp, Khichar *et al* (2014) reported fragments of 150 bp, 210 bp, 260 bp and 300 bp and Yadav *et al* (2015b) reported fragments of 80 bp, 150 bp, 210 bp and 300

Table 2. *Coa* gene variations in *S. aureus* isolates from camel skin wounds.

S. No.	Coagulase types	Isolates Numbers	Total isolates (%)	*coa* gene amplicon size (bp)
1.	1	C8, C12, C13, C22	4 (15.38)	510
2.	2	C4, C19	2 (7.69)	600
3.	3	C14, C15, C25, C26	4 (15.38)	680
4.	4	C1, C7, C9, C10, C16, C24	6 (23.07)	710
5.	5	C2, C3, C5, C6, C11, C17, C18, C20, C21, C23	10 (38.46)	760

Table 3. RFLP patterns of *coa* gene of *S. aureus* from camel skin wounds.

S.No.	RFLP Pattern	Isolates	Total isolates (%)	*coa* gene amplicon (bp)	RFLP fragment size (bp)
1	P I	C12, C13	2 (3.84%)	510	300, 210
2	P II	C8, C22	2 (3.84%)	510	210, 150, 80
3	P III	C4, C19	2 (3.84%)	600	300, 210, 80
4	P IV	C14, C15, C25, C26	4 (15.38)	680	260, 210
5	P V	C1, C7, C9, C10, C16, C24	6 (23.07)	710	300, 260, 150
6	PVI	C2, C3, C5, C6, C11, C17, C18, C20, C21, C23	10 (38.46)	760	760

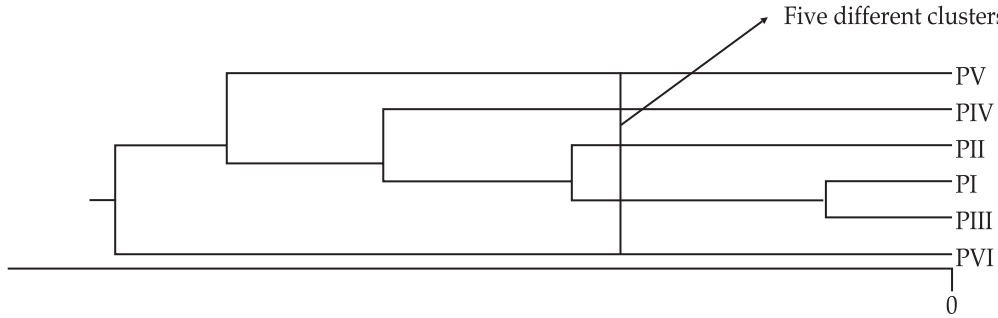

Fig 4. Cluster analysis of RFLP patterns of coa gene by dice and UPGMA method PI to PVI–different RFLP patterns.

bp. A variable RFLP patterns have been reported for *coa* amplicons with *S. aureus* isolates obtained from various origins. Schlegelova *et al* (2003) studied 86 *S. aureus* isolates from dairy cows and humans on a farm and from 10 different *coa* gene amplicon ranging between 650 bp to 1050 bp obtained 10 different RFLP pattern after *Alu*I digestion and their sizes varied from 80 bp to 490 bp.

In the present study *coa* amplicon of 760 bp was not digested with *Alu*I. This observation is in agreement with the findings of Lange *et al* (1999) who also did not observe digestion of three PCR products (580 bp, 650 bp and 1060 bp) from *S. aureus* of bovine mastitis origin. Similarly, da Silva and da Silva (2005) also observed non-digestion of two of the amplicons (579 bp and 602 bp) in isolates of cow mastitis origin. Yadav *et al* (2015b) also recorded non-digestion of one amplicon of 400 bp of mastitis isolates from cattle and buffalo.

Cluster Analysis of RFLP patterns

In the present investigation genetic relatedness among 6 RFLP patterns was calculated by Dice and UPGMA analysis method. Out of these 6 RFLP patterns 5 patterns were closely related of which pattern PI and PIII were closest. The maximum occurring (10 isolates) RFLP pattern PVI formed a separate cluster in the dendrogram (Fig 4).

In conclusion, the present study on 26 isolates of *S. aureus* from camel skin wound revealed that a great genetic difference existed among isolates in regard to both *spa* and *coa* genes. The genetic variability in *S. aureus* strains may be one of the reasons to exhibit difference in pathogenicity and refractory response towards treatment of *S. aureus* infections.

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