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With 6 tables in the text. www.sudanjvr.net

Isolation of Mycoplasmas and Sero-surveillance for *Mycoplasma mycoides* subsp. *mycoides* in the Dromedary Camel in Some Areas of the Sudan

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ملخص البحث

جمعت عينات أنسجة ومسحات لعزل المفطورات من 529 رأس من الإبل من ولايات الجزيرة ، كسلا والقضارف. كذلك جمعت عينات أمصال للكشف عن الأضداد للمفطورة الفطرانية تحت نوع الفطرانية من 603 راس من الإبل من ذات الولايات. عزلت معزولتين من أنسجة رئوية من الإبل من ولاية الجزيرة مصابة بإلتهاب رئوى ومعزولتين من مسحات أنفية لإبل من ولايات الجزيرة وكسلا وكانت أيضاً مصابة بالتهاب رئوى. عزلت أيضاً معزولة من مسحة أنفية لجمل بدأ صحيحاً في ولاية الجزيرة. بناءاً على الإختبارات التقليدية، فإن ثلاثة من المعزولات قد تنسب الى عدة أجناس من المفطورات واثنان من المعزولات قد تنسب الى جنس أكوليبلازما. ومن المثير للاهتمام، بأنه لم يتم عزل أى معزولة من ولاية القضارف. لقد عزلت المفطورات من ولايات الجزيرة وكسلا بنسبة 0.588% و 0.819% على التوالى وبنسبة عزل كلية بلغت 0.567%. فى حين أن الأكوليبلازما قد تم عزلها فقط من ولاية الجزيره بنسبة 0.588% وبلغت نسبة العزل الكلية 0.378%. لقد تم إكتشاف أضداد للمفطورة الفطرانية تحت نوع الفطرانية فى أمصال الإبل من الولايات الثلاثة بإستخدام إختبار التراص والمقايسة التنافسية للممتز المناعى المرتبط بالإنزيم، وكانت نسبة الكشف الكلية 9.62% و 3.81% على التوالى.

Summary

Tissue specimens and swabs were collected from 529 camels from El-Gezira, Kassala and Gedarif States for isolation and characterization of mycoplasmas. Serum samples for detection of antibodies to *Mycoplasma mycoides* subsp. *mycoides* small-colony (*Mmm*SC) in camel were collected from 603 camels from the aforementioned States. During meat inspection, two isolates were isolated from lung tissues from camels with pneumonic lungs in El-Gezira State, and two isolates from nasal swabs in Kassala and El-Gezira States. One isolate was from nasal swab from a camel with apparently normal lung in El-Gezira State. According to the conventional bacteriological and biochemical investigation three isolates were diagnosed as belonging to several genera of *Mycoplasma*, and two isolates as members of the genus *Acholeplasma.* Interestingly, no isolate was isolated from Gedarif State. The mycoplasmas were isolated from El-Gazera and Kassala States at rates of 0.588% and 0.819%, respectively, with an overall isolation rate of 0.567%. While acholeplasmas were only isolated from El-Gezira State at a rate of 0.588%, with overall isolation rate of 0.378%. Antibodies to *Mmm*SC antigen in camel sera were detected from the three states using slide agglutination test and c-ELISA, with an overall prevalence of 9.62% and 3.81%, respectively.

Introduction

The mycoplasmas are eubacteria that belonged to the class *Mollicutes* and are the smallest free-living and self-multiplying microorganisms that lack cell wall (Razin *et al.*, 1998). There are several clinically significant *mycoplamas* causing severe diseases and losses in ruminants in the Sudan as, *M. mycoides* subsp. *mycoides* small-colony (*Mmm*SC), the cause of contagious bovine pleuropneumonia (CBPP) in cattle (OIE, 2008a) and *M. capricolum* subsp. *capripneumoniae* (*Mccp*), the cause of contagious caprine pleuropneumonia (CCPP) in goats (OIE, 2008b). Neither of these diseases were reported in the dromedary camel either in the Sudan or elsewhere.

Camel mycoplasmosis was reported in a few limited cases. *M. arginini* was isolated in Saudi Arabia at the rate of 8.8% from pneumonic lungs of camels (Elfaki *et al.*, 2002) and in Egypt from clinically normal camels (Refai, 1990; 1992).

Other mycoplasmas were isolated from clinically normal camels include *Acholeplasma oculi* in Iraq (Al-Aubaidi *et al.*, 1978) and in Egypt (Refai, 1990; 1992). In addition *A. laidlawi* was also isolated in Egypt (Refai, 1990; 1992).

In the Sudan, the only report on camel mycoplasmas includes the isolation of unidentified *Mycoplasma* other than *Mmm*SC and isolation of acholeplasmas (ELNasri, 1977). Eisa (1985) reported isolation of *Mycoplasma* from pneumonic lungs of biochemical characters similar to *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M. verecundum*; and the isolation of *M. arginini* other than strain G230 from nasal swabs.

Sero-surveillance in clinical cases of pneumonia in the dromedary camel in Nigeria showed antibodies to *Mmm*SC by dot enzyme immunoassay and western blots at the rates of 12.1% and 6.8%, respectively (Egwu and Aliyu, 1997). However, in Kenya, antibodies to *Mmm*SC and *Mccp* were detected in clinically normal cases by complement fixation test at the rates of 6.8% and 62.7%, respectively (Paling *et.al.,* 1988). In the Sudan, no sero-surveillance attempts were carried out in the dromedary for either *Mmm*SC or *Mccp*.

The objectives of the present study were to isolate mycoplasmas from the dromedary camel in different localities in the Sudan, and to determine the prevalence of antibodies to *Mmm*SC in sera from the dromedary using slide agglutination test (SAT) and competitive - ELISA (c-ELISA).

Materials and Methods

Study area and samples collected:

Gezira, Kassala and Gedarif States were selected as study area. These states were selected on the basis of the high stocking density of camels and as assembly areas for camels from different localities of the Sudan during certain periods of the year. Tissue specimens and swabs were collected from 529 camels from the aforementioned states (Table 1). The tissues were placed in sterile plastic bags, while the swabs were placed in modified Hayflick medium (Freundt, 1983). Serum samples were collected from 603 camels from the same states (Table 2).

Culture media:

Six media were prepared; Modified Hayflick (Freundt, 1983), Brain Heart Infusion (Difco), H25P (Nicholas and Baker, 1998), Serum-free (Tully, 1984), SP-4 (Tully, 1995), and U9C (Shepard and Lunceford, 1976). The liquid media were dispensed in 5 ml aliquots and stored at 4°C until used within two weeks. The solid media were prepared by incorporation of Noble agar (Difco) in a concentration of 1.4% (w/v).

Mycoplasma isolation:

The tissue specimens and swabs were inoculated into Modified Hayflick and Brain Heart Infusion liquid media and incubated aerobically at 37ºC under humidified and 5% CO_2 tension for 3 hr, then 10-fold diluted (10^{-1} – 10^{-4}) in the same media, and re-incubated under the above mentioned conditions for up to two weeks. Evidence of growth was judged by opalescence or colour change compared to the un-inoculated control media. The grown culture was sub-cultured onto the corresponding solid media and incubated inverted in a sealed humid plastic container at 37ºC. The inoculated plates were examined under stereomicroscope every other day for typical fried egg or centre less colonies. The colonies were sub-cultured on fresh same media for adaptation. Swabs and tissue specimens that gave no growth in the former two media were

inoculated into SP-4, H25P and U9C media. The isolates were cloned following Tully (1983) and the viability was detected following Rodwell and Whitcomb (1983).

Preservation of viable isolates:

The isolates were preserved by sub-culturing in liquid and /or solid media and deep-freezing of 1 ml aliquots of young broth cultures or of approximately 0.5 cm^2 agar blocks containing young colonies at -20 ºC. For long-term preservation the young broth cultures were suspended in either equal volume of sterile 5% meso-inositol in horse serum (Redway and Lapage, 1974), or 2/5 volume of sterile 10% skimmed milk in dd H_2O before lyophilization. The Lyophilized cultures were stored at -20 ºC.

Table 1: Tissue specimens, sera and swabs collected from camels in three states in the Sudan.

Camel Description	Type of specimens	No. of samples collected/State			
		Gezira	**Kassala**	**Gedarif**	**Total**
apparently healthy camels in pasture	Nasal swabs	45	20	14	79
	Conjunctival swabs	45	20	14	79
	Vaginal swabs	18	6	2	26
	Sera	63	26	16	105
In pasture with nasal and/or occular discharges	Nasal swabs	29	12	7	48
	Conjunctival swabs	29	12	7	48
	Sera	29	12	7	48
In pasture, repeat breeder	Vaginal swabs	21	14	1	36
	Sera	21	14	1	36
With pneumonic lungs during meat inspection	Lung tissues	136	45	28	209
	Mediastinal and bronchopulmonary lymph nodes	136	45	28	209
	Bronchial swabs	136	45	28	209
	Tracheal swabs	136	45	28	209
	Nasal swabs	136	45	28	209
	Conjunctival swabs	136	45	28	209
Camels with normal lungs during meat inspection	Lung tissues	91	25	15	131
	Mediastinal and bronchopulmonary lymph nodes	91	25	15	131
	Bronchial swabs	91	25	15	131
	Tracheal swabs	91	25	15	131
	Nasal swabs	91	25	15	131
	Conjunctival swabs	91	25	15	131
Apparently normal prior slaughtering		241	73	42	356
With nasal and/or occular discharges prior slaughtering		34	11	13	58
Total		**728**	**258**	**146**	**1132**

Identification of isolates by biological, biochemical, and growth inhibition tests:

The cell morphology was identified by dark-field microscope, whereas the colonies were identified by Dienes stain (Dienes, 1945; Razin, 1983) and differentiated from the L-phase bacterial variants by pushed-agar-block technique (Razin, 1983) and reversion test. The isolates were differentiated according to digitonin sensitivity (Freundt *et al*. 1973), urea hydrolysis (Shepard and Howard, 1970), glucose fermentation, arginine hydrolysis, and phosphatase activity in colonies (Aluotto *et al.,* 1970), aesculin and arbutin hydrolysis (Williams and Wittler, 1971; Rose and Tully, 1983) and haemolysis of camel, sheep and guinea pig RBCs (Clyde, 1963; Aluotto *et al*. 1970). The growth inhibition test (Clyde, 1983) was implemented using filter paper disks impregnated with *Mmm*SC hyperimmune serum.

Detection of antibodies to *Mmm*SC in camel sera:

*Mmm*SC isolated from cattle was grown in 400 ml of modified Hayflick medium (Freundt, 1983), harvested by centrifugation in a cold centrifuge (+ 4ºC) at 12,000 *g* for 20 min, washed twice at the same conditions and re-suspended in 4 ml PBS before sonication. The sonicated suspension was used as *Mmm*SC antigen in the slide agglutination test (Priestly, 1951; Turner and Etheridge, 1963). The c-ELISA kit (Institut Pourquier/CIRAD) was used to detect the specific *Mmm*SC antibodies in the test camel sera following the producer's protocol.

Results

Isolation:

Five isolates were obtained. Table 2 shows camel description, anatomical location and designation of isolates. The biological and biochemical characters of isolates are shown in Table 3. All isolates were isolated in Modified Hayflick and Brain Heart Infusion media. The primary isolation of LEG98, LEG175 and NK43 took 7 days, while that of NEG175 and NEG36 took 6 days. When the isolates were adapted to the previous media by three consecutive sub-culturing, the growth was evident in 3-4 days. Negative specimens and swabs in Modified Hayflick and Brain Heart Infusion media also failed to grow in SP-4, H25P and U9C media when incubated for two weeks and they were considered negative. When horse serum in the media was replaced by camel serum, similar results were observed. All isolates were negative to disk growth inhibition test against cattle *Mmm*SC hyperimmune serum. The isolation rates of the different isolates from the three states are shown in Table 4.

Table 2: Camels description, anatomical location and designation of isolates

Camels description	Anatomical location	Isolate designation*
Camels with pneumonic lungs during meat inspection	Lung tissues	LEG98 and LEG175
	Nasal swabs	NK43
		NEG175
Camels with normal lungs during meat inspection	Nasal swabs	NEG36

** L = lung tissue; N = nasal swab; EG = El-Gezera State; K = Kassala State. The Arabic numbers indicate the sampled camel number. Isolates LEG175 and NEG175 were isolated from the same camel.*

Table 3: Biological and biochemical characters of isolates

	Preliminary biological characters								Biochemical characters								
		Colony morphology on solid media		Identification of mycoplasma colonies		Sterol requirement Digitonin sensitivity									Haemolysis		
				Differentiation of mycoplasma colonies from L-phase bacterial variants													
Isolate designation	Cell shape	Fried-egg shape	Dienes staining	Pushed-agar-block technique	Reversion test	Width of inhibition zone (mm)	Sensitivity	Growth in serum-free medium	Urea hydrolysis	Glucose fermentation	Arginine hydrolysis	Phosphatase activity	Aesculin hydrolysis	Arbutin hydrolysis	Camel RBCs	Sheep RBCs	Guinea pig RBCs
LEG98	P	+	+	FE	FE	6	S	NG	-	-	+	-	-	-	+	-	+
LEG175	P	+	+	FE	FE	5	S	NG	-	-	+	-	-	-	+	-	+
NK43	P	+	+	FE	FE	8	S	NG	-	-	+	-	-	-	+	-	+
NEG175	P	+	+	FE	FE	<2	R	G	-	+	-	±	+	+	+	+	+
NEG36	P	+	+	FE	FE	<2	R	G	-	+	-	±	+	+	+	+	+

P = pleomorphic; FE = fried-egg shape; S = sensitive; R = resistant; NG = no growth; G = growth; ± = weak positive.

Table 4: Isolation rates of the different isolates from Gezira, Kassala and Gedarif States.

	States			
	El-Gezira	**Kassala**	**Gedarif**	**Total**
No. of sampled camels	340	122	67	529
No. of isolates	2*	1**	0	3
Isolation rate	0.588%	0.819%	0%	0.567%
No. of isolates	2***	0%	0%	2
Isolation rate	0.588%	0%	0%	0.378%

** = Isolates LEG98 and LEG175; ** = Isolate NK43; *** = Isolates NEG175 and* NEG36.

Serological reaction of camel sera to *MmmSC* antigen:

The detection rates of *Mmm*SC antibodies in the camel sera from the three states by SAT and c-ELISA are shown in Table 5 and Table 6, respectively.

Table 5: Numbers and rates of camel sera reacting to *Mmm*Sc using slide agglutination test.

Camel description	State						Total	
	El-Gezera		Kassala		El-Gadaref			
	+/ No.	%	+/ No.	%	+/ No.	%	+/ No.	%
In pasture, clinically normal	11/63	17.46	1/26	3.85	1/16	6.25	13/105	12.38
In pasture, with nasal and/or ocular discharges	7/29	24.14	4/12	33.33	0/7	0	11/48	22.92
In pasture, repeat breeder	2/21	9.52	1/14	7.14	0/1	0	3/36	8.33
Apparently normal prior slaughter	14/241	5.81	3/73	4.11	2/42	4.76	19/356	5.34
With nasal and/or occular discharges prior slaughter	8/34	23.53	1/11	9.09	3/13	23.08	12/58	20.69
Total	**42/388**	**10.82**	**10/136**	**7.35**	**6/79**	**7.59**	**58/603**	**9.62**

+/No = Positive No./No. of sampled camels; % = percentage of positive sera.

Table 6: Numbers and rates of camel sera reacting to *MmmSc* using c-ELISA.

Camel description	State						Total	
	El-Gezera		Kassala		El-Gadaref			
	+/ No.	%	+/ No.	%	+/ No.	%	+/ No.	%
In pasture, clinically normal	6/63	9.52	1/26	3.85	0/16	0	7/105	6.67
In pasture, with nasal and/or ocular discharges	1/29	3.45	0/12	0	0/7	0	1/48	2.08
In pasture, repeat breeder	1/21	7.76	0/14	0	0/1	0	1/36	2.78
Apparently normal prior slaughter	8/241	3.32	3/73	4.11	1/42	2.38	12/356	3.37
With nasal and/or occular discharges prior slaughter	2/34	5.88	0/11	0	0/13	0	2/58	3.45
Total	18/388	4.64	4/136	2.94	1/79	1.27	23/603	3.81

+ No Positive; No.=No. of sampled camels; % = percentage of positive sera

Discussion

The camel population in the Sudan was estimated to be about 4,078,000 head (Anon, 2006). Camels constitute a considerable figure in the Sudan's gross domestic product, they used locally for transportation, as a source of red meat, milk prestige hide and hair, or exported for either meat consumption or camel racing. This study was carried out to study the role of mycoplasmas as possible pathogens affecting camel production in the Sudan.

During this study, five isolates were recovered from camels using conventional bacteriological methods. Based on the negative growth inhibition test using cattle *Mmm*SC hyperimmune serum, all isolates are not related to *Mmm*SC.

Based on sterol requirement, three of the isolates were sterol requiring and two were non-sterol requiring. Biochemically, all the sterol requiring isolates were glucose negative, arginine positive, phosphatase negative. This biochemical profile is similar to that of *M. arginini, M. canadense, M. columbinum, M. equirhinis, M. gallinarum, M. gateae, M. hominis, M. hyosynoviae, M. iners, M. lipophilum, M. orale, M. salivarium* and *M. subdolum* (Ernø, 1994). It was not possible to differentiate between them. However, the non-sterol requiring isolates are interpreted as *Acholeplasma* (Aluotto *et al.*, 1970; Williams and Wittler, 1971; Freundt *et al.*, 1973; Rose and Tully 1983) and the species could not be identified.

The three mycoplasma were isolated from camels with pneumonic lungs during meat inspection; two isolates from lung tissues and one isolate from nasal swab**.** However, both *Acholeplasma* isolates were isolated from nasal swabs during meat inspection; One isolate from a camel with pneumonic lungs and the other from normal camel lungs. It may be of interest to note that a *Mycoplasma* from lung and an *Acholeplasma* from a nasal swab were isolated from a camel with pneumonic lungs. The *Mycoplasma* species were isolated from El-Gezira and Kassala States. Whereas, members of the genus *Acholeplasma* were isolated from El-Gezera State only. Interestingly, neither *Mycoplasma* nor *Acholeplasma* was recovered from El-Gedarif State, this could be attributed to difference in camel husbandry, management or feeding.

It is evident that, the haemolytic activity is an important factor for virulence and survival of many mycoplasmas, which depends on the utilize sateen of RBCs (Aluotto *et al*., 1970). Failure of all the *Mycoplasma* isolates to haemolyse sheep RBCs could be attributed to the absence of the specific molecules in sheep RBCs that interact with the haemolysin(s) of these isolates.

The low isolation rate encountered during this study might have been due to presence of toxic or inhibitory substances in camel's tissue that hinder the cultivation of mycoplasmas (Taylor-Robinson and Chen, 1983; Tully and Whitcomb, 1992). Besides, that nutritional and environmental requirements of camel mycoplasmas are unknown and the used media may not support the isolation of fastidious mycoplasmas of camels. However, it could also be interpreted that, the isolated mycoplasmas grow rapidly to a point that they impede the recovery of fastidious mycoplasmas.

Serological reaction to test camel sera against *Mmm*SC antigen using SAT and c-ELISA indicated the presence of antibodies at rates of 9.62% and 3.81%, respectively. The difference in the detection rate of the two tests could be attributed to the

fact that, the later test uses a specific monoclonal antibody that reacts with *Mmm*Sc antibodies only.

In this study, three *Mycoplasma* isolates and two *Acholeplasma* isolates were recovered, besides the detection of antibodies to the highly infectious *Mmm*SC that affects cattle and causes severe losses. These results could point out that the dromedary camel may act as carrier.

As it was not possible to discriminate between the *Mycoplasma* isolates at the genus level, and the *Acholeplasma* isolates at the species level, molecular techniques are recommended for certain identification of the isolates from the conserved 16S rRNA genes (Kai *et al*., 1991; Van Kuppeveld *et al*., 1992; Dussurget and Roulland-Dussoix, 1994). Pathogenecity studies of the dromedary camel for significant *Mmm*SC of cattle in the Sudan are warranted.

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