The Sudan J. Vet. Res. (2012), 27:7-14.
With 6 tables 2 figures in the text. *www. Sudanjvr.net.*

Amplification and Sequencing of the 16S rRNA Genes of Some *Mycoplasma* Isolates from the Dromedary Camel in the Sudan

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ملخص البحث

لقد عزلت سابقاً ثلاث مفطورات واثنتان أكوليبلازما من الإبل فى السودان بإستخدام الطرق التقليدية. لقد تم توصيف هذه المعزولات خلال الدراسة الحالية على مستوى الجنس والنوع بواسطة تكثير جينات 16 اس للرنا الرايبوزي باستخدام تفاعل البلمرة التسلسلي ثم تبع ذلك تسلسل هذه النواتج. لقد دل تحليل التسلسل الى ان المفطورات تنتمى الى المفطورة الأرجنينية بينما الاكوليبلازما تنتمى الى أكوليبلازما اليدلاوية.

Summary

Three *Mycoplasma* and two *Acholeplasma* isolates were previously isolated from the dromedary camel (*Camelus dromedarius*) in the Sudan by conventional bacteriological methods (Elghazali, 2011). In this study, the isolates were further characterized at the genus and species levels by amplification of their 16S rRNA genes by polymerase chain reaction (PCR) using genus- and species-specific primer pairs, and by sequencing of the amplicon fragments. Sequence analysis indicated that, the *Mycoplasma* isolates belong to *Mycoplasma arginini* while the *Acholeplasma* isolates belong to *Acholeplasma laidlawii*.

Introduction

The mycoplasmas are eubacteria belonging to the class *Mollicutes*. They 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 among these are, *Mycoplasma 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). None of these diseases were reported in the dromedary camel in the available literature worldwide.

Camel mycoplasmosis was reported in a few cases. *M. arginini* was isolated in Saudi Arabia from pneumonic lungs (Elfaki *et al.*, 2002) and from clinically normal camels in Egypt (Refai, 1990; 1992). Other mycoplasmas isolated from clinically normal camels include, *Acholeplasma oculi* in Iraq (Al-Aubaidi *et al.*, 1978) and in Egypt (Refai, 1990; 1992) and *A. laidlawi* in Egypt (Refai, 1990; 1992).

In the Sudan, the only report on camel mycoplasmas included the isolation of unidentified *Mycoplasma* other than *Mmm*SC and isolation of Acholeplasmas (ELNasri, 1977). Further, Mycoplasmas from pneumonic lungs with biochemical activities similar to *M. agalactiae*, *M. bovis*, *M. bovigenitalium* and *M. verecundum* were reported and *M. arginini* other than G230 strain was isolated from nasal swabs (Eisa, 1985). Recently, five isolates were obtained by conventional bacteriological methods (Elghazali, 2011); four of which were from camels with pneumonic lesions; two isolates from lung tissues (isolates nos. LEG98 and LEG175 from El-Gezera State), and the other two from nasal swabs (isolates nos. NK43 and NEG175 from Kassala and El-Gezera states, respectively). The fifth isolate was isolated from a nasal swab from a camel with apparently normal lung during meat inspection (isolate no. NEG36 from El-Gezera State). Interestingly, the Isolates nos. LEG175 and NEG175 were recovered from the same camel. Based on the conventional bacteriological methods, the isolates nos. LEG98, LEG175 and NK43 were related to several species of *Mycoplasma* including *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), whereas isolates nos. NEG175 and NEG36 were correlated to the genus *Acholeplasma* (Aluotto *et al*., 1970; Williams and Wittler, 1971; Freundt *et al.*, 1973; Rose and Tully 1983).

The 16S rRNA conserved gene sequence is one of the markers used in the phylogeny of *Mollicutes* (Woese *et al.*, 1980) and is recommended by the International Committee on Systematic Bacteriology-Subcommittee in the Taxonomy of *Mollicutes* (1997).

There are several, sensitive molecular techniques used to characterize mycoplasmas at the genus and species levels, including amplification of the 16S rRNA genes by polymerase chain reaction (PCR) using genus-specific and species-specific primers (Kai *et* al., 1991; Van Kuppeveld *et al*., 1992; Dussurget and Roulland-Dussoix, 1994).

The objective of the present study was to characterize previously recovered *Mycoplasma* isolates nos. LEG98, LEG175, NK43, NEG175 and NEG36 at the genus and species levels, by amplification of their 16S rRNA genes by PCR using genus- and species-specific primers and sequencing of the amplified products.

Materials and methods

Isolates:

Mycoplasma isolates nos. LEG98, LEG175, NK43, NEG175 and NEG36 were previously isolated from dromedary in the Sudan (Elghazali, 2011), were used in this study.

Genomic DNA extraction and purification from the isolates:

The DNA was extracted by the Phenol/Chloroform method following Bashiruddin (1998) and its quality and quantity was assessed by a single-beam spectrophotometer before storage at -20°C for further examination.

Agarose Gel Electrophoresis:

A minigel 8 cm long electrophoresis unit was used. The reagents included 1.5% (w/v) agarose gel (SeaKem LE Agarose; Cambrex) in 0.5x Tris-Borate-EDTA (TBE; 45 *mM* Tris-borate/1 *mM* EDTA, pH 8.3) buffer, 1 µg/ml ethidium bromide in ddH_2O, TBE running buffer, Blue/Orange 6X Loading Dye (Promega), 1 Kb Plus (Invitrogen) and 100 bp Plus (Vivantis) DNA size makers. The bands were visualized and photographed by a gel documentation system.

Amplification of the 16S rRNA gene by polymerase chain reaction (PCR):

PCR reagents:

The *Taq* DNA polymerase enzyme (Invitrogen) was accompanied by 10X PCR Buffer Minus Mg and 50 mM $MgCl_2$ in separate vials. The dNTPs (Vivantis) were supplied in a set of 100 mM aqueous solution of dATP, dCTP, dGTP, and dTTP, each in a separate vial. Two types of oligonucleotide primers were synthesized (Bioneer Corporation) as illustrated in Table 1, genus-specific (RNA5, RNA3 and UNI-) and species-specific (ARG2 and ACH3) primers as described by Kai *et* al. (1991), Van Kuppeveld *et al*. (1992) and Dussurget and Roulland-Dussoix (1994).

Table 1: Primer designation and sequence

Primer specificity	Primer designation	Primer sequence (5' → 3')
Genus-specific	RNA5 (forward)	AGAGTTTGATCCTGGCTCAGGA
	RNA3 (reverse)	ACGAGCTGACGACAACC**R**TGCAC
	UNI- (reverse)	TAATCCTGTTTGCTCCCCAC
Species-specific	ARG2 (reverse)	TCAACCAGGTGTTCTTTCCC
	ACH3 (forward)	AGCCGGACTGAGAGGTCTAC

The ambiguity code '**R**' in the RNA3 (reverse) primer sequence indicates 'A or G' according to the International Union of Biochemistry (IUB)

The 1st PCR reaction using RNA5 and RNA3 primers:

A 50 µl/200 µl PCR tube reaction mix was prepared and composed of sterile ddH_2O, 18.75 µl; 10X PCR buffer minus Mg, 5 µl; 10 mM dATP, 1 µl; 10 mM dCTP, 1 µl; 10 mM dGTP, 1 µl; 10 mM dTTP, 1 µl; 10 µM RNA5 primer, 2.5 µl; 10 µM RNA3 primer, 2.5 µl; *Taq* DNA Polymerase (5 U/µl), 0.25 µl; 50 mM $MgCl_2$, 1.5 µl; and genomic DNA template, 5 µl. A thermocycler (Isogen Life Science; peqlab Primus 96 advanced) with a heated lid was used and adjusted to initial denaturation cycle at 95°C for 2 min, amplification for 30 cycles consisting of denaturation at 95°C for 45 sec, annealing at 55°C for 1 min and extension at 72°C for 1.5 min, additional extension cycle at 72°C for 10 min, and hold at 4°C. A volume of 5 µl from each PCR amplicon was analysed by agarose gel electrophoresis using a small comb. The rest of the amplicom was loaded in the agarose gel wells made by a large comb and the well-separated DNA bands were excised and the

DNA was extracted using GF-1 Gel DNA Recovery Kit (Vivantis). The purified fragments are used as templates in the later 2nd PCR reactions and as inserts in the pCR®II-TOPO vector of the TOPO TA Cloning kit (Invitrogen).

The 2nd PCR reaction using RNA5 and ARG2 primers:

The reaction mix was prepared in a similar fashion as the previous mix except that the RNA3 primer was replaced with ARG2 primer, sterile ddH_2O was increased to 20.75 µl and the 5 µl genomic DNA template was replaced with 3 µl of the agarose gel extracted DNA fragment. The thermocycling conditions were similar to the previous 1st PCR conditions except, that the initial denaturation cycle was reduced to 94°C for 1.5 min. A volume of 5 µl from each PCR amplicon was analysed by agarose gel electrophoresis using a small comb. The rest of the amplicom was processed for gel extraction, as previously described in the 1st PCR, to use the fragments as inserts in the pCR®II-TOPO vector of the TOPO TA Cloning kit (Invitrogen).

The 2nd PCR reaction using ACH3 and UNI- primers:

The reaction mix was prepared in a similar fashion as the previous mix except that the RNA5 was replaced with ACH3 and the ARG2 primer was replaced with the UNI- primer. The thermocycling conditions were similar to the previous 2nd PCR conditions. The amplicon was processed as the previous 2nd PCR amplicon.

Cloning of the gel purified PCR fragments:

The gel purified fragments from the 1st and the 2nd PCR products were cloned using the TOPO TA Cloning kit (Invitrogen) and following the producer's protocol.

Sequencing of the cloned PCR inserts and analysis of the sequences:

The recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (QIAGEN) and following the supplied protocol. A volume of 30 µl (100 ng/µl) of the recombinant plasmids were sent to Macrogen Inc. for automated bidirectional sequencing of the 1st PCR amplicons and unidirectional sequencing of the 2nd PCR amplicons using the pCR®II-TOPO cloning vector primers (M13 forward and M13 reverse). The sequences were analyzed by the BioEdit software package and the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov).

Results

Agarose gel electrophoresis of the 1st and 2nd PCR reactions:

The 1st PCR of the five isolates using the genus-specific primer pairs RNA5/RNA3 gave amplicon fragments of about 1000 bp (Fig. 1). The 2nd PCR of the LEG98, LEG175 and NK43 isolates using the species-specific primer pairs RNA5/ARG2 gave amplicon fragments of between 400 and 500 bp (Fig. 2). The 2nd PCR of the NEG175 and NEG36 isolates using the species-specific primer pairs ACH3/UNI- gave amplicon fragments nearly similar to those of the previous three isolates (Fig. 2).

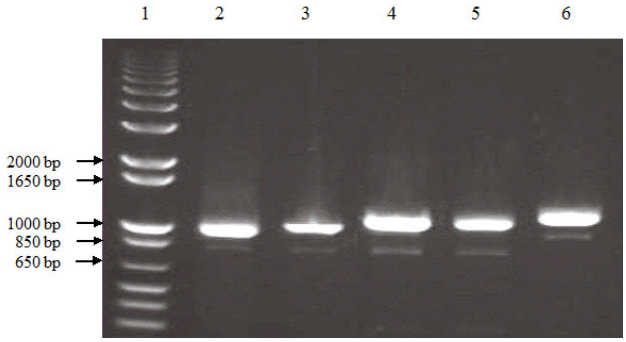

Fig. 1: 1st PCR using the genus-specific primer pairs RNA5/RNA3.
Line 1 = 1 Kb Plus DNA Ladder (Invitrogen); line 2 = isolate NEG175; line 3 = isolate NEG36; line 4 = isolate LEG98; line 5 = isolate LEG175; line 6 = isolate NK43

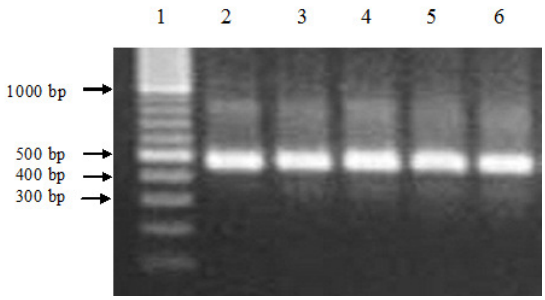

Fig. 2: 2nd PCR using the species-specific primer pairs ACH3/UNI- and RNA5/ARG2.
Line 1 = 100 bp Plus DNA Ladder (Vivantis); lines 2 and 3 = isolate NEG175 and NEG36, respectively using the species specific primer pairs ACH3/UNI-; lines 4, 5 and 6

Sequences of the 1st PCR-amplicons:

The amplicons of the LEG98, LEG175 and NK43 isolates were similar in sequence, of about 1047 bp in length, and belonged to *M. arginini* (Table 2). The amplicons of the NEG175 and NEG36 isolates were similar in sequence, of about 1027 bp in length, and belonged to *A. laidlawii* (Table 3).

Table 2: BLAST analysis of the 1st PCR sequences of LEG98, LEG175 and NK43

Accession No.	Description	Query coverage	Max. ident.
GQ409971.1	*M. arginini* strain 284F08 16S rRNA gene, partial sequence.	99%	99%
HM179556.1	*M. arginini* strain EF-Hungary 16S rRNA gene, partial sequence; 16S-23S rRNA intergenic spacer, complete sequence; and 23S rRNA gene, partial sequence.	98%	99%
U15794.1	*M. arginini* 16S rRNA gene, partial sequence.	97%	99%
AF125581.1	*M. arginini* strain G230(T) 16S rRNA gene, partial sequence.	96%	99%
M24579.1	*M. arginini* 16S rRNA small subunit.	94%	99%
HM635904.1	*M. arginini* strain C1-Beh-10 16S rRNA gene, partial sequence.	67%	99%

Table 3: BLAST analysis of the 1st PCR sequences of NEG175 and NEG36

Accession No.	Description	Query coverage	Max. ident.
CP000896.1	*A. laidlawii* PG-8A, complete genome.	100%	100%
M23932.1	*A. laidlawii* 16S rRNA small subunit gene.	100%	99%
FJ226559.1	*A. laidlawii* strain SRCD 16S rRNA gene and 16S-23S rRNA intergenic spacer, partial sequence.	97%	99%
EU925161.1	*A. laidlawii* strain REP 16S rRNA gene and 16S-23S rRNA intergenic spacer, partial sequence.	97%	99%
FJ590758.1	*A. laidlawii* strain TTB 103 16S rRNA gene and 16S-23S rRNA intergenic spacer, partial sequence.	97%	99%
FJ655561.1	*A. laidlawii* strain CIRG/Alw-1 16S rRNA gene, partial sequence.	97%	99%
NR_025961.1	*A. laidlawii* strain PG8 16S rRNA, partial sequence. U14905.1|ALU14905 *A. laidlawii* PG8 ATCC 23206 16S rRNA gene, partial sequence.	100%	98%
FJ226570.1	*A. laidlawii* strain Haig 179L 16S rRNA gene and 16S-23S rRNA intergenic spacer, partial sequence.	97%	99%
AM073014.1	*A. laidlawii* 16S rRNA gene.	68%	99%

Sequences of the 2nd PCR-amplicons:

The amplicons of the LEG98, LEG175 and NK43 isolates wre similar in sequence, of about 450 bp in length, and belonged to *M. arginini* (Table 4). The amplicons of the NEG175 and NEG36 isolates were similar in sequence, of about 485 bp in length, and belonged to *A. laidlawii* (Table 5).

Table 4: BLAST analysis of the 2nd PCR sequences of LEG98, LEG175 and NK43

Accession No.	Description	Query coverage	Max. ident.
M24579.1	*M. arginini* 16S rRNA small subunit.	100%	99%
GQ409971.1	*M. arginini* strain 284F08 16S rRNA gene, partial sequence.	99%	99%
HM179556.1	*M. arginini* strain EF-Hungary 16S rRNA gene, partial sequence; 16S-23S rRNA intergenic spacer, complete sequence; and 23S rRNA gene, partial sequence.	95%	99%
U15794.1	*M. arginini* 16S rRNA gene, partial sequence.	94%	99%
AF125581.1	*M. arginini* strain G230(T) 16S rRNA gene, partial sequence.	92%	99%
HM635904.1	*M. arginini* strain C1-Beh-10 16S rRNA gene, partial sequence.	23%	99%

Table 5: BLAST analysis of the 2nd PCR sequences of NEG175 and NEG36

Accession No.	Description	Query coverage	Max. ident.
FJ655561.1	*A. laidlawii* strain CIRG/Alw-1 16S rRNA gene, partial sequence.	100%	100%
FJ590758.1	*A. laidlawii* strain TTB 103 16S rRNA gene and 16S-23S rRNA intergenic spacer, partial sequence.	100%	100%
FJ226570.1	*A. laidlawii* strain Haig 179L 16S rRNA gene and 16S-23S rRNA intergenic spacer, partial sequence.	100%	100%
FJ226559.1	*A. laidlawii* strain SRCD 16S rRNA gene and 16S-23S rRNA intergenic spacer, partial sequence.	100%	100%
EU925161.1	*A. laidlawii* strain REP 16S rRNA gene and 16S-23S rRNA intergenic spacer, partial sequence.	100%	100%
CP000896.1	*A. laidlawii* PG-8A, complete genome	100%	100%
M23932.1	*A. laidlawii* 16S rRNA small subunit gene.	100%	100%
NR_025961.1	*A. laidlawii* strain PG8 16S rRNA, partial sequence. U14905.1|ALU14905 *A. laidlawii* PG8 ATCC 23206 16S rRNA gene, partial sequence.	100%	98%
AM073014.1	*A. laidlawii* 16S rRNA gene.	88%	99%

Isolation rates:

Based on the conventional and the molecular characterization, the isolation rates of *M. arginini* and *A. laidlawii* from different anatomical and geographical locations are illustrated in Table 6.

Table 6: Isolation rates of *M. arginini* and *A. laidlawii*

Description		El-Gezera	Kassala	El-Gadaref	Total
No. of sampled camels		340	122	67	529
M. arginini isolates	No. of isolates	2[a]	1[b]	0	3
	Isolation rate	0.588%	0.819%	0%	0.567%
A. laidlawii isolates	No. of isolates	2[c]	0%	0%	2
	Isolation rate	0.588%	0%	0%	0.378%

[a] Isolates LEG98 and LEG175; [b] Isolate NK43; [c] Isolates NEG175 and NEG36

Discussion

Based on the conventional bacteriological methods three *Mycoplasma* isolates (LEG98, LEG175 and NK43) and two *Acholeplasma* isolates (NEG175 and NEG36) were obtained (Elghazali, 2011). The present study was implemented to characterize the five isolates from their conserved 16S rRNA genes. The 16S rRNA gene sequences have indicated that the *Mycoplasma* isolates belonged to *M. arginini* whereas the *Acholeplasma* isolates belonged to *A. laidlawii*.

Both *M. arginini*, and *A. laidlawii* are among the limited number of isolated *Mollicutes* from camels. In addition to *M. arginini* (Eisa, 1985; Refai, 1990; 1992; Elfaki *et al.*, 2002) and *A. laidlawii* (Refai, 1990; 1992), *A. oculi* (Al-Aubaidi *et al.*, 1978; Refai, 1990; 1992) and un-identified *Mycoplasma* (ELNasri, 1977; Eisa, 1985), were also isolated from camels and identified by only conventional bacteriological methods. Among these isolates, only *M. arginini* (Elfaki *et al.*, 2002) was isolated from pneumonic lung tissues and the rest were isolated from clinically normal camels.

Both *M. arginini* and *A. laidilawii*, have been isolated from different anatomicall sites, respiratory system, urogenital tract, udder, conjunctiva and ear canal, from clinically normal animals including cattle (Gourlay *et al.*, 1979; Jasper, 1981; Doig and Ruhnke, 1986), sheep and goats (Goltz *et al.*, 1986; Rosendal, 1994; Ayling *et al.*, 2004; Gonçalves *et al.,* 2010), swines (Whittlestone, 1979; Ross, 1986), dogs (Kirchholff, 1973; Eberle *et al.*, 1977; Rosendal, 1982), cats (Rosendal, 1979), and horses (Lemke, 1979; Tully, 1985) and were usually considered, in all of these animal species, to be non-pathogenic microorganisms.

In the present study, the three *M. arginini* isolates were isolated from camels with pneumonic lungs during meat inspection; two isolates from lung tissues and one isolate from a nasal swab**.** However, both *A. laidilawii* isolates were recovered from nasal swabs during meat inspection; one isolate from camel pneumonic lungs and the other from camel normal lungs. Besides, *M. arginini* from lung and *A. laidilawii* from a nasal swab were isolated from one camel with pneumonic lungs. This may be contravential to the general notion which considers both *M. arginini* and *A. laidilawii* to be non-pathogenic in camel (Refai, 1990; 1992) and in other animal species (Ayling *et al.*, 2004; Doig and Ruhnke, 1986; Eberle *et al.*, 1977; Goltz *et al.*, 1986; Gonçalves *et al.,* 2010; Gourlay *et al.*, 1979; Jasper, 1981; Kirchholff, 1973; Lemke, 1979; Rosendal, 1979; 1982; 1994; Ross, 1986; Tully, 1985; Whittlestone, 1979). However, this study agrees with that described by Elfaki *et al.* (2002) in isolation of *M. arginini* from pneumonic lungs of camels.

Screening of *M. arginini* and *A. laidlawii* for genes or DNA sequence(s), acquired by lateral gene transfer from other micro-organisms, that could be associated with pathogenecity, is highly recommended. It is also recommended to conduct pathogenecity studies on the dromedary camel for *M. arginini* and *A. laidlawii*.

Acknowledgements

The authors would like to thank the former Director of the Central Laboratory, Prof. Abdulrahim Mohamed Hussein for providing a great opportunity to work at his laboratory, the former Director of the Veterinary Research Institute, Prof. Amal Mustafa Mohammed Ali for her support throughout the present study, the director of the Veterinary Research Institute and the Director General of Animal Resources Research Corporation for permission to publish this article.

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