Various Inc-type plasmids and lineages of Escherichia coli and Klebsiella pneumoniae spreading blaCTX-M-15, blaCTX-M-1 and mcr-1 genes in camels in Tunisia

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Short Communication

Various Inc-type plasmids and lineages of *Escherichia coli* and *Klebsiella pneumoniae* spreading $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}1}$ and *mcr-1* genes in camels in Tunisia

Mariem Saidani[a,b], Lilia Messadi[a], Jomâa Mefteh[a], Amira Chaouechi[a], Alya Soudani[a], Rachid Selmi[a], Monia Dâaloul-Jedidi[a], Faten Ben Chehida[a], Aymen Mamlouk[a], Mohamed Habib Jemli[c], Jean-Yves Madec[d], Marisa Haenni[d,*]

[a] Service de Microbiologie et d'Immunologie, Ecole Nationale de Médecine Vétérinaire, Université de La Manouba, Sidi Thabet, Tunisia
[b] Institut National Agronomique de Tunisie, Université de Carthage, Tunis, Tunisia
[c] Service de Parasitologie, Ecole Nationale de Médecine Vétérinaire, Université de La Manouba, Sidi Thabet, Tunisia
[d] Unité Antibiorésistance et Virulence Bactériennes, Université de Lyon - ANSES Site de Lyon, Lyon, France

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ABSTRACT

Objectives: Resistance to extended-spectrum cephalosporins, fluoroquinolones and colistin is under constant scrutiny in food-producing animals worldwide. However, little is known about camels, which provide milk and meat for human consumption, and are attractions for tourists to ride in arid regions. This study assessed the role of camels as potential reservoirs of these resistance determinants.
Methods: Faecal swabs were collected from 232 camels in Tunisia between April 2016 and July 2018. Enterobacteriaceae were detected on MacConkey agar and extended-spectrum β-lactamase (ESBL)-producers on the same medium supplemented with cefotaxime. Antimicrobial resistance was assessed by disc diffusion, and ESBL-producing isolates were further characterised by phylogrouping (for *Escherichia coli, E. coli*) and multilocus sequence typing. Genetic support of the bla_{ESBL} and *mcr-1* genes was identified by plasmid-typing and Southern blot.
Results: E. coli were identified in 163 of 232 (70.3%) and *Klebsiella pneumoniae* (*K. pneumoniae*) in 16 of 232 (6.9%) of the dominant flora. Three *E. coli* and one *K. pneumoniae* (1.3% and 0.4%, respectively) were found on cefotaxime-enriched media. One *K. pneumoniae* and one *E. coli* from a tourist farm harboured the $bla_{CTX\text{-}M\text{-}15}$ gene on an IncY plasmid, while the two *E. coli* from the butchery sector displayed the $bla_{CTX\text{-}M\text{-}15}$ gene on an IncI1 plasmid and colocalisation of the $bla_{CTX\text{-}M\text{-}1}$ and *mcr-1* genes on an IncHI2 plasmid.
Conclusions: This study reported ESBL-producing Enterobacteriaceae in Tunisian camels from both tourist and meat-producing sectors. This was the first description of the *mcr-1* gene in a meat-producing camel. Although not alarming, this context needs specific attention to avoid camels becoming a bigger reservoir for multidrug-resistant Enterobacteriaceae.
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1. Introduction

The burden of multidrug-resistant (MDR) Enterobacteriaceae in animals is under constant scrutiny because of the risk of transmission of resistance determinants to humans, either through food consumption or by direct contact with animals. Particular attention has been paid to resistances to specific antimicrobial families, such as broad-spectrum cephalosporins and fluoroquinolones, which are molecules of critical importance for human health. Colistin has also recently been under the spotlight as it is widely used in animals yet is a last-resort antibiotic in humans, especially to treat infections due to carbapenemase-producing pathogens. In the animal sector, the proportions and types of resistance to these major antibiotics have been extensively documented in poultry, swine and bovines, which are the main meat-producing animals worldwide, as well as in cats, dogs and horses, which are common domestic animals [1].

Little is known about resistance determinants in camels, despite their important economic value in arid lands as they provide meat, milk, wool and leather; they are also a tourist

* Corresponding author at: Unité Antibiorésistance et Virulence Bactériennes, Université de Lyon - ANSES Site de Lyon, 31 Avenue Tony Garnier, 69364, Lyon, France.
E-mail address: marisa.haenni@anses.fr (M. Haenni).

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attraction for riding in desert regions. Camels are characterised by their ability to adapt to adverse environmental conditions such as a lack of water and pasture. Thanks to their hardiness, camels are also naturally resistant to many diseases commonly affecting livestock. Nevertheless, camels, and camel calves in particular, also suffer from infectious diseases that need to be treated by antibiotics. Only a few studies have characterised resistance determinants in camels: one publication reported high resistance proportions of extended-spectrum β-lactamase (ESBLs) in *Pseudomonas aeruginosa* from camel meat in Egypt due to the presence of PER-1 and CTX-M enzymes [2]. A high proportion of ESBL-producing *Escherichia coli* (*E. coli*) (five of 26, 19.2%) was reported in Saudi Arabia [3]. In Tunisia, three studies lead to divergent results, with two publications reporting the absence of ESBLs and colistin-resistance in camel calves [4,5], and a third one identifying a single CTX-M-1-producing *E. coli* isolate recovered from a healthy adult dromedary [6].

To clarify the role of camels as a potential reservoir of ESBLs and colistin-resistance in Tunisia, this study looked for Enterobacteriaceae in the gut flora of 232 healthy camels. It selectively detected and characterised ESBL-producing *E. coli* and *Klebsiella pneumoniae* (*K. pneumoniae*) isolates, of which one was additionally resistant to colistin.

2. Material and methods

2.1. Sampling and isolation of bacteria

From April 2016 to July 2018, faecal swabs were collected from 232 camels originating from the south (n = 136), coast (n = 80) or centre-east (n = 16) of Tunisia (Table 1). The 135 females and 97 males that were sampled were raised extensively or intensively for meat production, except one herd that was raised semi-intensively for tourist purposes in a zoo located on the Mediterranean coast (Table 1). Faecal samples were transported to the microbiology laboratory in ice-cooled containers and immediately processed. Each swab was incubated for 18–24 h at 37 °C in buffered peptone water for enrichment. Bacterial cultures (10 μL) were then inoculated on MacConkey agar plates without and with cefotaxime (2 μg/mL) to screen for dominant Enterobacteriaceae and the sub-dominant ESBL-producing Enterobacteriaceae, respectively. Isolates were identified by classical biochemical methods. For each faecal sample, only one representative of each bacterial species was kept for further studies. Bacterial isolates were suspended in brain heart infusion with 20% glycerol and stored at −80 °C until further processing.

2.2. Antimicrobial susceptibility testing and ESBL identification

Antimicrobial susceptibility for *E. coli* and *K. pneumoniae* to 10 β-lactam (amoxicillin, amoxicillin/clavulanic acid, cefalexin, cefoxitin, ceftiofur, cefepime, cefotaxime, ceftazidime, aztreonam and ertapenem) and six non-β-lactam (chloramphenicol, gentamicin, nalidixic acid, ciprofloxacin, tetracycline and sulfonamide/trimethoprim) antibiotics was tested by the disk diffusion method on Mueller-Hinton agar. The inhibition zone was measured and compared with interpretative diameters according to the clinical breakpoints recommended by the Antibiogram Committee of the French Society of Microbiology (CA-SFM; www.sfm-microbiologie.org). The ESBL phenotype was screened by double disk synergy test. Resistance to colistin was searched for all ESBL-producing isolates by the ColiSpot test, as described by Jouy et al. [7] and the MIC values of ColiSpot-positive isolates were determined by the broth microdilution method. The *E. coli* ATCC 25922 strain was used as the quality control.

2.3. Detection of ESBL and colistin-resistance genes

The presence of β-lactamase genes was detected using a CTX-M group-specific multiplex PCR [8]. For the CTX-M-1 group, an additional PCR was performed using external primers (ISEcp1L1, 5'-CAGCTTTTATGACTCG and P2D, 5'-CAGCGCTTTTG CCGTCTAAG) and all amplicons were sequenced. Colistin-resistance genes were detected by PCR, as recently described [9].

2.4. Phylogenetic grouping and multilocus sequence typing

The detection of *E. coli* phylogenetic groups (A, B1, B2 or D) was determined by PCR, as described by Doumith et al. [10]. The MLST of ESBL isolates was determined according to protocols described on the *E. coli* (https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef) and *K. pneumoniae* MLST websites (http://bigsdb.pasteur.fr/klebsiella/primers_used.html).

2.5. Plasmid analysis of ESBL-producing and mcr-1-producing isolates

Plasmids were typed by PCR-based replicon typing (PBRT) according to the PBRT kit scheme, described by Carattoli et al., using a commercial kit (Diatheva, Fano, Italy) [11]. Plasmids carrying the ESBL genes were detected using pulse-field gel electrophoresis performed on DNA digested by the S1 endonuclease (PFGE-S1; 6 V/cm for 20 h with a 120° angle at 14 °C with pulse times ranging from 1–30 s) [12]. After electrical transfer of the DNA on Nylon® membrane, Southern blots were performed according to the manufacturer's protocol using adequate probes (Roche Diagnostics, Meylan, France). After overnight hybridisation at 37 °C, all membranes were treated with maleic acid and blocking solution for 45 min, then with purified immunoglobulin Anti-Dioxigenin-AP Fab Fragments®, Washing Buffer and finally coloured with NBT/BCIP. Plasmid colocalisation was assessed by comparison between the bands corresponding to the resistance gene and those corresponding to the Inc type of the plasmid.

Table 1
Description of the sampling.

Region	City	Number of samples (n = 232)	Type of farming	Farming destination
South	Douz	66	Extensive	Butchery
	Médenine	3	Extensive	Butchery
	Tataouine	36	Extensive	Butchery
	Tozeur	25	Extensive	Butchery
	Kébili	6	Extensive	Butchery
Coast	Sousse	39	Semi-intensive	Tourism
	Sousse	13	Intensive	Butchery
	Mahdia	5	Intensive	Butchery
	Bouficha	23	Intensive	Butchery
Centre	Kairouan	16	Intensive	Butchery

3. Results and discussion

A total of 257 Enterobacteriaceae isolates were recovered from 232 camel faecal swabs. The frequencies of the Enterobacteriaceae species grown on non-selective plates were 163 of 232 (70.3%) for *E. coli* and 16 of 232 (6.9%) for *K. pneumoniae*. Of note, one representative of each bacterial species was studied per faecal sample, so that all *E. coli* or all *K. pneumoniae* originated from different animals. Other Enterobacteriaceae were also isolated, namely: *Citrobacter* spp. (n = 29), *Enterobacter* spp. (n = 16), *Escherichia fergusonii* (n = 7), and *Salmonella enterica* (n = 6). The 20 remaining isolates could not be reliably identified and were thus discarded from the study. Phylogenetic analysis revealed that *E. coli* isolates belonged to the phylogroups A (n = 67, 41.1%), B1 (n = 74, 45.4%), B2 (n = 6, 3.7%) or D (n = 16, 9.8%), suggesting the predominance of commensal isolates (phylogroups A and B1).

Antimicrobial resistance rates in *E. coli* and *K. pneumoniae* isolates recovered from the dominant flora are listed in Table 2, with the highest resistance rate observed for tetracyclines in both species (27.0% and 31.3%, respectively). The globally high proportion of tetracycline resistance in Tunisian camels may be due to the wide use of this antibiotic to treat animals in this country. One *E. coli* (0.6%) was resistant to fluoroquinolones (ciprofloxacin), while this resistance was absent in *K. pneumoniae*. Resistance rates were lower than those observed by Bessalah et al. where, for example, the resistance rate to tetracyclines was 52.8% [4]. These variations may result from differences in the studied animal populations. The current study focused on healthy adults only, while Bessalah et al. also partially investigated diarrhoeic camel calves. As observed for livestock in many countries, the inclusion of sick, and thus possibly treated, animals in the sampling design may have globally increased the antimicrobial resistance rates in this study.

The proportion of Enterobacteriaceae isolates recovered from the subdominant flora on the cefotaxime-enriched medium was four of 232 (1.7%) when taken globally, and proportions per bacterial species were three of 232 (1.3%) for *E. coli* and one of 232 (0.4%) for *K. pneumoniae*. These data are in accordance with previously published results where the only CTX-M-1-producing *E. coli* recovered from a camel in Tunisia had also been recovered on a selective medium [4,6]. Two isolates originated from a zoo (*E. coli* D147 and *K. pneumoniae* D157) and one from a butcher's farm *E. coli* B11) located in the coastal area of Tunisia. The last ESBL-producing *E. coli* (T4) was isolated from a butcher's farm in the south of Tunisia. For this last isolate only, the ColiSpot test revealed the presence of colistin-resistance, which was confirmed by broth microdilution (MIC value of 6 mg/L); PCR analysis and sequencing on this isolate revealed the presence of the *mcr-1* gene. This is the first detection of the plasmid-mediated colistin-resistance *mcr-1* gene in camels since a previous study in Tunisia dedicated to the detection of *mcr* genes lead to negative results [5]. All four ESBL-producing isolates presented multiple associated resistances to non-β-lactam antibiotics (Table 3). These data prove that ESBL-producing Enterobacteriaceae can be isolated in camels from both tourist and meat-producing sectors, and that farming processes are not the only context for the presence or absence of ESBL-producing Enterobacteriaceae in camels.

Table 2
Antimicrobial resistance in *E. coli* and *K. pneumoniae* isolates collected from camel faeces.

Antibiotics	*E. coli* (n = 163)		*K. pneumoniae* (n = 16)	
	Number	%	Number	%
Amoxicillin	16	9.8	–	–[a]
Amoxicillin + clavulanic acid	6	3.7	4	25.0
Cefalexin	15	9.2	3	18.8
Cefoxitin	0	0.0	0	0.0
Ceftiofur	0	0.0	0	0.0
Cefepime	0	0.0	0	0.0
Cefotaxime	0	0.0	0	0.0
Ceftazidime	0	0.0	0	0.0
Aztreonam	0	0.0	0	0.0
Ertapenem	0	0.0	0	0.0
Chloramphenicol	9	5.5	1	6.3
Gentamicin	6	3.7	0	0.0
Nalidixic acid	5	3.1	1	6.3
Ciprofloxacin	1	0.6	0	0.0
Tetracycline	44	27.0	5	31.3
Sulfonamide/trimethoprim	6	3.7	0	0.0

Abbreviations: *E. coli*, *Escherichia coli*; *K. pneumoniae*, *Klebsiella pneumoniae*.
[a] intrinsic resistance.

In this context, an important issue is the true prevalence of ESBL carriage in camels' guts, which surely varies from one farm to another, as observed for other food-producing animal species. In 2012, Ben Sallem et al. reported a 50% prevalence of ESBL carriage in camels, albeit measured on two animals. The current data (1.3% in *E. coli* and 0.4% in *K. pneumoniae*), obtained on a large collection of 232 camels from various geographical origins, instils confidence that ESBL prevalence in healthy camels in Tunisia is most probably rather low [6]. The current study also presented much lower rates of ESBL-producing isolates that in Saudi Arabia (five of 26, 19.2%); however, in this latter study, the ESBL phenotype was suggested by Vitek-2 analysis only and not molecularly confirmed, so any comparison of ESBL rates should be considered with caution [3]. Interestingly, two of the four ESBL-producing isolates were found among 39 camels reared in a zoo. Further investigations are needed to explore whether camels with a closer link to human activities may be more prone to having a higher prevalence of ESBL gut carriage than camels reared in other contexts.

Multilocus sequence typing revealed that the two ESBL-carrying isolates originating from a zoo were a ST224 *E. coli* and ST983 *K. pneumoniae*. In both isolates, the ESBL phenotype was due to the presence of the $bla_{\text{CTX-M-15}}$ gene carried by an IncY plasmid. The simple hypothesis of an interspecies transmission of a unique IncY plasmid is not supported by the current data, since those plasmids did not present the same size (170 and 40 kbp, respectively). However, a first transmission event followed by distinct molecular evolutions of the IncY plasmid within each bacterial host cannot be excluded. Human to animal transmission through close contact in a zoological park may also be plausible since the $bla_{\text{CTX-M-15}}$ gene is much more frequently identified in humans than in animals. Nonetheless, the ST983 *K. pneumoniae* lineage is rather rare and is not a typical human clone. Conversely, *E. coli* ST224 has been identified in humans and often in animals, as reported in swine in Brazil, in cats in Brazil and France, or in ducks in China [13–16]. At this stage, any hypothesis of intersectoral transmission of the $bla_{\text{CTX-M-15}}$ gene remains speculative.

The two remaining ESBL-producing *E. coli* were isolated from camels raised for butchery. One ST10 *E. coli* presenting the $bla_{\text{CTX-M-15}}$ gene on an IncI1 plasmid was detected in the coastal region of Tunisia. IncI1 plasmids have long been associated with wide dissemination of the $bla_{\text{CTX-M-1}}$ gene in animals, including in Tunisia [17], but they are now commonly detected in humans and the environment. Likewise, the ST10 clone has a globally-distributed lineage with no host specificity. The last ST162 *E. coli* was isolated from the south of Tunisia and harboured the $bla_{\text{CTX-M-1}}$ and *mcr-1* genes encoding for the ESBL and colistin-resistance phenotype on the same IncHI2 plasmid. The IncHI2 plasmid is among one of the most prevalent plasmids spreading *mcr-1* worldwide. Notably, colocalisation of *mcr-1* with $bla_{\text{CTX-M-1}}$ on IncHI2 plasmids has been reported in veal calves in France and also in chickens in Tunisia [18]. Detection of the *mcr-1*/$bla_{\text{CTX-M-1}}$ IncHI2 plasmid in camels reflects its global epidemiological success. Of note, the *mcr-1*/$bla_{\text{CTX-M-1}}$ combination has also been identified in

Table 3
Characteristics of ESBL-producing isolates.

Isolate	Species	ST[a]	Phylo-group	Geographical origin	Rearing context	Associated resistances[b]	bla_{ESBL} and *mcr* genes	Plasmid size (kbp)	Plasmid type
D147	*E. coli*	224	A	Coast	Zoo	TET, NAL, SXT	$bla_{CTX\text{-}M\text{-}15}$	40	IncY
D157	*K. pneumoniae*	983	–	Coast	Zoo	GEN, TET, NAL, SXT	$bla_{CTX\text{-}M\text{-}15}$	170	IncY
B11	*E. coli*	10	A	Coast	Butchery	NAL, CIP, TET, SXT	$bla_{CTX\text{-}M\text{-}15}$	90	IncI1
T4	*E. coli*	162	B1	South	Butchery	NAL, CIP, TET, SXT, COL	$bla_{CTX\text{-}M\text{-}1}$, *mcr-1*	260	IncHI2

Abbreviations: *E. coli*, *Escherichia coli*; *K. pneumoniae*, *Klebsiella pneumoniae*
[a] ST: sequence type.
[b] Antibiotics: NAL, nalidixic acid; CIP, ciprofloxacin; TET, tetracycline; SXT, sulfonamide-trimethoprim; COL, colistin.

other animal hosts and countries [19]. In addition, the same ST162 *E. coli* clone was reported to carry the *mcr-1* gene in black vultures in Spain [20].

In conclusion, this study reports the occurrence of CTX-M-15- and CTX-M-1-producing Enterobacteriaceae, and the first description of the *mcr-1* gene in camels in Tunisia. These resistant bacteria were isolated from both tourist and meat-producing sectors, and the bacterial lineages that were identified could not be clearly assigned to the animal or human reservoir. Various Inc-type plasmids were responsible for spreading these genes, including the IncHI2 plasmid already known as a major vehicle of *mcr-1* and $bla_{CTX\text{-}M\text{-}1}$ worldwide. Consequently, even though the low prevalence of ESBLs and colistin resistance is not yet an alarming issue in camels, vigorous monitoring is needed to avoid any further spread in this animal group that is of major economic importance in North African countries. Indeed, camels may act as potential reservoirs for acquiring resistance genes from humans and the environment, but also for re-infecting humans who are in contact with them.

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Competing interest

None.

Ethical approval

Not required.

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