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Extended-Spectrum β-Lactamase CTX-M-15-Producing *Klebsiella pneumoniae* of Sequence Type ST274 in Companion Animals

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Screening of extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria in companion animals living in the Paris area in France identified a high rate of CTX-M-15-producing *Klebsiella pneumoniae*. Those isolates were recovered during the 2010-2011 period from both infections and asymptomatic colonizations. Sequence typing revealed that most of these isolates belonged to sequence type ST274. Interestingly, the $bla_{CTX\text{-}M\text{-}15}$ gene was located on a specific and novel plasmid scaffold. These findings highlight that companion animals may be reservoirs for CTX-M-15-producing *K. pneumoniae* evolving separately from the human reservoir of CTX-M-15 producers.

Multidrug resistance in bacteria isolated from animals is an emerging phenomenon, mirroring what is actually observed among humans (1). In particular, resistance to broad-spectrum cephalosporins is increasingly reported not only in food-producing animals but also in domestic animals (2, 3). It is speculated that animals, including domestic pets, may be reservoirs of multidrug-resistant bacteria. Although the prevalence of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* is reaching alarming rates worldwide in humans (4), the emergence of ESBL producers in animals also raises some important concerns (1, 5). The ESBL CTX-M-15 is considered the most common ESBL identified worldwide in humans (3). Whereas ESBL-producing enterobacterial isolates (and in particular CTX-M-1 producers) have been quite often reported in animals, the occurrence of CTX-M-15 producers in animals has been very limited so far (1, 6). In France, both CTX-M-1 and CTX-M-15 were identified from *Escherichia coli* isolates recovered from food-producing animals (7). In addition, a recent study performed in the United States on animal isolates showed that CTX-M-15 was identified only in *E. coli* (8). Apart from the occurrence of ESBL producers, some recent studies identified carbapenemases in Gram-negative bacteria from animals, i.e., OXA-23-producing *Acinetobacter* genomospecies 15TU from dairy cattle in France (9), OXA-23-producing *Acinetobacter* spp. from horses in Belgium (10), and VIM-1-producing *E. coli* isolates from pigs and poultry in Germany (11, 12). Our study aimed to evaluate the occurrence of ESBL-producing or carbapenemase-producing *Enterobacteriaceae* as commensals (rectal isolates) or as pathogens (urinary tract infections) among companion animals living in France.

During the period between July 2011 and June 2012, screening of companion animals ($n = 90$) together with animals considered wild fauna (birds, geese, and hedgehogs) ($n = 20$) was undertaken by performing rectal and cloacal swabs at the Veterinary School of Maisons-Alfort, in the suburbs of Paris, France. The companion animals were mainly cats and dogs but also included three sheep living in close contact with humans and considered companion animals in that case. The wild animals have been sampled for the purpose
of that study. The wildlife center, which is a separate building from that dealing with domestic animals, receives injured (trauma), sick (parasitism or cachexia), or orphaned wild animals, all of them found in urban or periurban areas of the Paris suburbs. Note that those wild animals have been hospitalized at the Veterinary School. Samples were precultured in buffered peptone-water and incubated for 18 h at 37°C. Cultures were inoculated by streaking 100 μl of the suspensions onto ChromID ESBL agar plates (bioMérieux, La Balmes-les-Grottes, France) to select for ESBL-producing isolates and onto Drigalski plates containing 30 μg/ml of imipenem to select for carbapenem-resistant Gram-negative isolates. In addition, a total of 105 enterobacterial isolates recovered from urine specimens only from dogs and cats were collected.

Identification of isolates at the species level was performed by using the API20E system (bioMérieux, La Balme-les-Grottes, France). Susceptibility testing was performed by disk diffusion assay (Sanofi-Diagnostic Pasteur, Marnes-la-Coquette, France), and MICs were determined by Etest (bioMérieux) on Mueller-Hinton agar plates at 37°C and interpreted according to the CLSI guidelines (13). Production of ESBL was evaluated by double-disk synergy testing and confirmed by the ESBL NDP test (14).

Thirty-three isolates exhibiting an ESBL phenotype were recovered from the screening, most of them being recovered from the rectal screening of the domestic animals ($n = 20$) and others being recovered from urine of cats and dogs ($n = 9$) and from wild fauna ($n = 4$). Overall, the ESBL producers were from dogs ($n = 19$), cats ($n = 7$), sheep ($n = 3$), domestic goose ($n = 1$), European hedgehog ($n = 1$), rock pigeon ($n = 1$), and tawny owl ($n = 1$).

The ESBL-producing isolates were distributed as follows: *Klebsiella pneumoniae*, $n = 15$; *E. coli*, $n = 15$; *Klebsiella oxytoca*, $n = 2$; and *Escherichia fergusonii*, $n = 1$ (Table 1). During that overall screening, no enterobacterial isolate exhibiting reduced susceptibility to carbapenems (ertapenem and imipenem) was recovered.

Received 28 December 2012 **Returned for modification** 13 January 2013
Accepted 10 February 2013
Published ahead of print 19 February 2013
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doi:10.1128/AAC.02622-12

TABLE 1 Features of the ST274 *K. pneumoniae* clinical isolates

Isolate[a]	Bacterial species	Animal	Sample	CTX-M	TEM	DHA-1	Sequence type
Kp1	*K. pneumoniae*	Hedgehog	Rectal	CTX-M-15	TEM-1	+	ST274
Kp2	*K. pneumoniae*	Dog	Rectal	CTX-M-15	TEM-1	+	ST274
Kp3	*K. pneumoniae*	Dog	Rectal	CTX-M-15	TEM-1	+	ST274
Kp4	*K. pneumoniae*	Sheep	Rectal	CTX-M-15	TEM-1	+	ST274
Kp5	*K. pneumoniae*	Sheep	Rectal	CTX-M-15	TEM-1	+	ST274
Kp6	*K. pneumoniae*	Sheep	Rectal	CTX-M-15	TEM-1	+	ST274
Kp7	*K. pneumoniae*	Dog	Rectal	CTX-M-15	TEM-1	+	ST274
Kp8	*K. pneumoniae*	Dog	Rectal	CTX-M-15	TEM-1	+	ST274
Kp9	*K. pneumoniae*	Dog	Rectal	CTX-M-15	TEM-1	+	ST274
Kp10	*K. pneumoniae*	Dog	Urine	CTX-M-15	TEM-1	−	ST15
Kp11	*K. pneumoniae*	Cat	Urine	CTX-M-15	TEM-1	+	ST274
Kp12	*K. pneumoniae*	Dog	Urine	CTX-M-15	TEM-1	+	ST274
Kp13	*K. pneumoniae*	Dog	Urine	CTX-M-15	TEM-1	+	ST274
Kp14	*K. pneumoniae*	Cat	Urine	CTX-M-15	TEM-1	+	ST274
Kp15	*K. pneumoniae*	Cat	Urine	CTX-M-15	TEM-1	+	ST274
Ec1	*E. coli*	Tawny owl	Rectal	CTX-M-1		−	ST93
Ec2	*E. coli*	Domestic goose	Rectal	CTX-M-15	TEM-1	−	ST10
Ec3	*E. coli*	Rock pigeon	Rectal	CTX-M-1		−	ST124
Ec4	*E. coli*	Dog	Rectal	CTX-M-1		−	ST345
Ec5	*E. coli*	Dog	Rectal	CTX-M-1		−	ST1001
Ec6	*E. coli*	Dog	Rectal	CTX-M-15		−	New ST[c]
Ec7	*E. coli*	Dog	Rectal	CTX-M-1		−	New ST[c]
Ec8	*E. coli*	Dog	Rectal		TEM-52	−	ST359
Ec9	*E. coli*	Dog	Rectal	CTX-M-1		−	ST124
Ec10	*E. coli*	Dog	Rectal	CTX-M-1		−	ST124
Ec11	*E. coli*	Cat	Rectal	CTX-M-1		−	ST124
Ec12	*E. coli*	Dog	Rectal	CTX-M-1		−	ST124
Ec13	*E. coli*	Cat	Rectal	CTX-M-1		−	ST641
Ec14	*E. coli*	Dog	Urine	CTX-M-1		−	ST345
Ec15	*E. coli*	Cat	Urine	CTX-M-14		−	ST141
Ko1	*K. oxytoca*[b]	Dog	Rectal	CTX-M-15	TEM-1	−	ND[d]
Ko2	*K. oxytoca*[b]	Dog	Urine	CTX-M-15	TEM-1	−	ND
Ef1	*E. fergusonii*	Sheep	Rectal	CTX-M-1		−	ND

[a] Isolates Ec4 and Kp2 are from a single dog; isolates Kp4 and Ko1 are from a different single dog.
[b] The two *K. oxytoca* isolates were from two different dogs.
[c] The two new STs are different from each other.
[d] ND, not determined.

Detection of ESBL and plasmid-borne AmpC-encoding genes (bla_{CMY}, bla_{ACC}, and bla_{DHA}) was carried out by PCR (5). Purified PCR products were then sequenced on both strands using an Applied Biosystems sequencer (ABI 377). Four types of ESBLs were identified among the 33 ESBL-positive isolates: CTX-M-15 ($n = 19$), CTX-M-1 ($n = 12$), CTX-M-14 ($n = 1$), and TEM-52 ($n = 1$). The last three ESBLs have been frequently identified among animal isolates in Europe (1). However, CTX-M-15, though already reported, is not considered a frequent ESBL among animals, in contrast to what is observed among humans. All CTX-M-15-producing *K. pneumoniae* isolates except one coproduced the TEM-1 penicillinase in addition to the plasmid-mediated AmpC DHA-1 (Table 1).

Since 15 out of the 19 CTX-M-15-positive isolates corresponded to *K. pneumoniae*, genotyping was performed. The first approach was made by multilocus sequence typing (MLST), performed as described previously (15); it surprisingly showed that all but one of the *K. pneumoniae* isolates belonged to ST274, the exception being isolate Kp10, typed as ST15 and recovered from dog urine (Table 1). The clonal relationship of the *K. pneumoniae* ST274 isolates was further investigated by repetitive extragenic palindromic sequence PCR (rep-PCR) using the DiversiLab system dedicated for *Klebsiella* (bioMérieux) according to the manufacturer's recommendations. It revealed that those 13 strains actually corresponded to two main clones, each of them including isolates recovered from different animal species (Table 1). All the ST274 isolates were resistant to tetracycline, gentamicin, nalidixic acid, sulfonamides, and trimethoprim-sulfamethoxazole. In addition, six isolates were resistant to ofloxacin and tobramycin. All remained susceptible to ciprofloxacin, nitrofurantoin (except one), amikacin, and netilmicin. A single isolate recovered from dog urine was additionally resistant to nitrofurantoin and chloramphenicol.

In order to evaluate whether ST274 *K. pneumoniae* strains could also be widespread among humans, we randomly selected a total of 20 $bla_{CTX\text{-}M\text{-}15}$-positive *K. pneumoniae* isolates recovered at the Bicêtre hospital (located in the Paris area, like the veterinary school) during the same period of time, half being from urinary tract infections and half being from rectal samples taken as part of routine screening. MLST analysis showed that no ST274 isolate was identified among those 20 isolates, which were distributed among 15 distinct ST types. This indicated a difference in clonal epidemiology between the animal and human CTX-M-15-positive *K. pneumoniae* isolates. Even if it appears very unlikely, we

cannot rule out the possibility of a local spread of the ST274 strain at the veterinary school. However, according to our results, it seems very unlikely that the ecology observed among the animals might be considered the reflection of the human ecology. The literature appears to show a single report of ST274 *K. pneumoniae* in humans so far, corresponding to KPC-2-producing carbapenem-resistant strains isolated in Greece in the 2009-2010 period (16). We also found a single ST15 CTX-M-15-producing *K. pneumoniae* isolate recently identified in France from pets (17).

Among the 12 CTX-M-1-producing isolates, 11 were actually *E. coli*, and MLST performed as described previously (18) identified six different STs, ST124 ($n = 5$) being the main one and the others being ST10 ($n = 1$), ST93 ($n = 1$), ST345 ($n = 2$), ST641 ($n = 1$), and ST1001 ($n = 1$) (Table 1).

Mating assays were performed using the CTX-M-15-positive *K. pneumoniae* and CTX-M-1 *E. coli* isolates as donors and azide-resistant *E. coli* J53 as the recipient strain, as described previously (5). All *E. coli* transconjugants exhibited a resistance pattern in accordance with the expression of an ESBL, and PCR assays confirmed that they were indeed expressing CTX-M-15 or CTX-M-1, respectively. Plasmid analysis performed by using the Kieser extraction method (19) revealed that all $bla_{\text{CTX-M-15}}$-positive *E. coli* transconjugants harbored a single plasmid which was estimated to be ca. 250 kb according to a size marker. PCR-based replicon typing (PBRT) was performed as described previously (20) to identify the incompatibility groups of the $bla_{\text{CTX-M-1}}$- and $bla_{\text{CTX-M-15}}$-bearing plasmids, respectively. It showed that the $bla_{\text{CTX-M-1}}$ gene was always located on an IncI1-type plasmid, which is in accordance with previous studies (3, 21, 22). The $bla_{\text{CTX-M-15}}$-positive *E. coli* transconjugants could not be typed by PBRT, giving negative results for all plasmid groups tested. Analysis of the susceptibility patterns of those *E. coli* transconjugants showed that they were resistant to tetracycline, gentamicin, sulfonamides, and trimethoprim-sulfamethoxazole (Table 1). Notably, these *E. coli* transconjugants also possessed the $bla_{\text{TEM-1}}$ and $bla_{\text{DHA-1}}$ genes. The occurrence of the bla_{AmpC} gene was not expected since the *E. coli* transconjugants, similarly to the donor *K. pneumoniae* isolates, did not show resistance to cefoxitin, a common resistance marker associated with the expression of many plasmid-mediated AmpC β-lactamases. This might be due to a poor expression of the corresponding gene in those donor and recipient strains.

Conclusion. Currently, the ESBL CTX-M-15 is considered to have spread worldwide and to be the most common source of resistance to broad-spectrum cephalosporins in *E. coli* and to a lesser extent in *K. pneumoniae* (23). Whereas human infections with multidrug-resistant CTX-M-15-producing *K. pneumoniae* isolates are mainly a nosocomial problem, CTX-M-15-producing *E. coli* strains are disseminated widely in the community. The present study aimed to evaluate whether companion animals could represent a reservoir of strains harboring those plasmids in France. Surprisingly, we found a high rate of CTX-M-15-producing *K. pneumoniae* in those animals. However, they did not carry the $bla_{\text{CTX-M-15}}$ gene on an IncFII-type plasmid, which is by far the main vehicle of $bla_{\text{CTX-M-15}}$ transmission in human isolates (21), but interestingly, this gene was identified on a novel plasmid scaffold which was widely distributed in different animal species. Further studies are in progress to characterize this plasmid. Interestingly, even if finding CTX-M-1-producing *E. coli* was somehow to be expected from taking into consideration published data on the subject, the frequent occurrence of CTX-M-15-producing *K. pneumoniae* that we observed here allowed us to identify an unexpected reservoir for those clinically relevant multidrug-resistant isolates which is likely evolving in parallel with the human reservoir.

ACKNOWLEDGMENTS

This work was partially funded by a grant from the INSERM (U914), by the Ministère de l'Education Nationale et de la Recherche (UPRES-EA3539), by Université Paris XI, France, and mostly by grants from the European Community (TEMPOtest-QC, HEALTH-2009-241742; R-GNOSIS, FP7/HEALTH-F3-2011-282512; and MAGIC-BULLET, FP7/HEALTH-F3-2001-278232). L.P. was funded by a grant-in-aid from the Ecole Nationale Vétérinaire de Maisons-Alfort (ENVA) through an INSERM-ENVA contract.

We thank C. Bouillin and C. Gandoin (LCAAST) for their technical assistance.

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