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Marisa Haenni, Estelle Saras, Véronique Métayer, Christine Médaille, Jean-Yves Madec. High Prevalence of blaCTX-M-1/IncI1/ST3 and blaCMY-2/IncI1/ST2 Plasmids in Healthy Urban Dogs in France. *Antimicrobial Agents and Chemotherapy*, 2014, 58 (9), pp.5358 - 5362. 10.1128/aac.02545-14. anses-04018309

HAL Id: anses-04018309

<https://hal-anses.archives-ouvertes.fr/anses-04018309>

Submitted on 7 Mar 2023

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Antimicrob. Agents Chemother. 2014, 58(9):5358. DOI:
10.1128/AAC.02545-14.
Published Ahead of Print 30 June 2014.

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High Prevalence of *bla*_{CTX-M-1}/IncI1/ST3 and *bla*_{CMY-2}/IncI1/ST2 Plasmids in Healthy Urban Dogs in France

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In the community, close contacts between humans and dogs may promote the transfer of extended-spectrum beta-lactamase/plasmidic AmpC cephalosporinase (ESBL/pAmpC) genes. Large-scale prevalence studies on ESBL/pAmpC carriage in dogs are rare, and data on ESBL/pAmpC plasmids are even more limited. Here, a considerable rate of 18.5% ESBL/pAmpC carriers was found among 368 unrelated healthy dogs in Paris, France. This prevalence is much higher than the one found in healthy humans in the same city (6%) but close to that recently reported in dogs in China (24.5%). All isolates were identified as *Escherichia coli*, except one *Salmonella enterica* and one *Klebsiella pneumoniae* isolate. The sequence type 131 (ST131) clone was rare (2/73 isolates). Interestingly, two plasmids (*bla*_{CTX-M-1}/IncI1/ST3 and *bla*_{CMY-2}/IncI1/ST2) were unexpectedly highly predominant, raising the question of their successful spread. Considering that CTX-M-1 was recently found to be equally as abundant as CTX-M-15 in healthy Parisian subjects, the question of dogs being a CTX-M-1 reservoir for humans is open. Such a high prevalence of the *bla*_{CMY-2}/IncI1/ST2 plasmid may result from the use of cephalosporins in veterinary medicine, as previously demonstrated experimentally. In all, our study points out healthy urban dogs as a potential source of ESBL/pAmpC genes that can further disseminate to the human community.

Extended-spectrum beta-lactamases (ESBL) and plasmidic AmpC cephalosporinases (pAmpC) are the two main mechanisms conferring resistance to expanded-spectrum cephalosporins in Gram-negative bacteria. In particular, ESBLs of the CTX-M-type and the pAmpC CMY-2 were increasingly reported worldwide, and livestock or companion animals are potential sources of β -lactam-resistant bacteria in humans (1, 2). If the use of expanded-spectrum cephalosporins in animals undoubtedly favors the emergence and dissemination of the corresponding genes, the prominent routes—and directions—of transfer in animals or between animals and humans are still debated.

ESBL/pAmpC genes were shown to spread efficiently in association with specific clones, such as the *Escherichia coli* sequence type 131 (ST131) clone carrying the *bla*_{CTX-M-15} gene (3). ST131 has also been detected occasionally in animals, notably companion animals, possibly reflecting owner-to-pet transmission (4). However, most studies highlighted the existence of different *E. coli* bacteria harboring *bla*_{CTX-M}- or *bla*_{CMY-2}-carrying plasmids, some of which are shared between humans and animals. Thus, increasingly the data suggest that plasmids may play an even greater role than clonal dissemination in the spread of these genes (5, 6).

In companion animals, ESBL/pAmpC producers and plasmids have been much less reported than in food animals. Moreover, most studies were carried out on infectious samples (7–10), and the real prevalence in healthy companion animals can be questioned due to the limited size of the collections investigated so far (11–13). Large sets of data are also lacking on the ESBL/pAmpC plasmids circulating in healthy pets, which is of major importance with respect to the risk of transfer to humans in daily situations of close contact.

This work is the largest prevalence study of ESBL/pAmpC-producing *Enterobacteriaceae* and subsequent ESBL/pAmpC plasmid analysis in healthy dogs worldwide. We also highlight a surprising distribution of these plasmids in the studied population of dogs.

MATERIALS AND METHODS

Animals screened, bacterial isolation, and identification. From March 2011 to December 2011, fecal specimens from 368 nonduplicate consecutive healthy dogs, all belonging to different owners (as attested by different names and home addresses), were sampled at the same veterinary clinic in a suburb of Paris. Owners were from different parts but mostly from south of the capital. Dogs had no clinical symptoms and were admitted for routine physical examination, parasite screening, or vaccination procedures. Samples were directly plated onto selective ChromID ESBL plates (bioMérieux, Marcy l'Etoile, France) to select for ESBL- and AmpC-producing (chromosomal and plasmidic) *Enterobacteriaceae*. Presumptive colonies of *Enterobacteriaceae* were collected (two colonies per presumptive species), and identification was performed using colony morphology and API galleries (bioMérieux). All natural pAmpC producers and non-*Enterobacteriaceae* isolates (mostly *Pseudomonas* spp.) were discarded.

Antibiotic susceptibility testing. Susceptibility testing was performed by disc diffusion according to the guidelines and clinical breakpoints of the Antibiogram Committee of the French Society for Microbiology (www.sfm-microbiologie.fr). Susceptibility to 32 antibiotics of veterinary and human interest was tested (Table 1; see also Table S1 in the supplemental material), and ESBL production was confirmed by a double-disc synergy test (see Table S1). *E. coli* ATCC 25922 was used as a quality control. The inhibitory effect of cloxacillin on AmpC production was observed on plates supplemented with 200 mg/liter cloxacillin (AES Chemunex, Bruz, France).

Received 14 February 2014 Returned for modification 15 March 2014

Accepted 22 June 2014

Published ahead of print 30 June 2014

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.02545-14>.

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doi:10.1128/AAC.02545-14

TABLE 1 Non-β-lactam resistance associated with CMY-2- or ESBL-producing *E. coli*

Antibiotic	Zone diam breakpoint (mm) ^a		No. of associated resistances (proportion [%]) in:		Comparison of proportions (<i>P</i>)
	S≥	R<	CMY-2-producing <i>E. coli</i> (<i>n</i> = 20)	ESBL-producing <i>E. coli</i> (<i>n</i> = 47)	
Streptomycin	15	13	7 (35)	13 (28)	0.55
Kanamycin	17	15	8 (40)	10 (21)	0.11
Amikacin	17	15	0 (0)	0 (0)	
Apramycin	15	12	0 (0)	0 (0)	
Gentamicin	18	16	7 (35)	4 (9)	0.02
Tobramycin	18	16	6 (30)	6 (13)	0.18
Netilmicin	21	19	0 (0)	3 (6)	
Chloramphenicol	22	19	11 (55)	8 (17)	0.002
Florfenicol	19	15	0 (0)	1 (2)	
Tetracycline	19	17	13 (65)	29 (62)	0.80
Colistin	18	15	0 (0)	0 (0)	
Trimethoprim	16	12	13 (65)	23 (49)	0.23
Sulfonamides	17	12	14 (70)	35 (74)	0.71
Nalidixic acid	20	15	13 (65)	21 (45)	0.13
Enrofloxacin	22	17	13 (65)	13 (28)	0.004
Ofloxacin	22	22	13 (65)	13 (28)	0.004

^a S, susceptible; R, resistant.

Beta-lactamase and cephalosporinase gene identification. The *bla*_{CTX-M} genes were detected using a CTX-M group-specific multiplex PCR, while the *bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA}, and *bla*_{CMY-2} genes were screened by simplex PCRs (14–16). For the CTX-M-1 group, an additional PCR was performed using external primers (ISEcp1L1, 5'-CAGCTTTTATGA CTCG; P2D, 5' CAGCGCTTTTGCCGTCTAAG). All ISEcp1L1/P2D-positive amplicons were sequenced (Beckman Coulter, London, United Kingdom).

Transferability of the *bla*_{ESBL/pAmpC} genes and plasmid characterization. Plasmids were transferred by conjugation to *E. coli* rifampin-resistant K-12 J53 recipient strains and replicon typed using PCR-based replicon typing (PBRT) scheme (17). The sizes of plasmids were determined by S1 pulsed-field gel electrophoresis (PFGE) gels (15). Southern blot hybridizations were performed with a *bla*_{CTX-M} or *bla*_{CMY-2} probe and probes corresponding to the various replicon types found (15), either on transconjugants when successfully obtained or on native strains. Plasmid subtypes were determined using plasmid multilocus sequence typing (pMLST) for IncI1 (<http://pubmlst.org/plasmid/>) or the replicon sequence typing (RST) scheme for IncF (18, 19).

Typing of the bacteria. Pulsed-field gel electrophoresis (PFGE) was performed using the restriction enzyme XbaI. Phylogenetic grouping of the *E. coli* isolates was performed as described previously (20). The B2–O25b–ST131 clone of *E. coli* was detected using the PCR-based assay described by Clermont et al. (21), with a human *E. coli* isolate of the B2–O25b:H4–ST131 clonal group included as a positive control (courtesy of Marie-Hélène Nicolas-Chanoine, Paris, France). Multilocus sequence typing (MLST) was carried out on the presumptive ST131 isolates according to the protocol described on the *E. coli* MLST website (<http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>).

Statistical analyses. Comparisons of proportions were done using a chi-square test (with Yates's correction when needed). The significance level was set to 0.05.

RESULTS AND DISCUSSION

This study is the largest one investigating the digestive colonization rate of ESBL/pAmpC producers in dogs worldwide. Of 368 dogs tested, 68 (18.5%) were positive for ESBL/pAmpC production on selective plates. As four fecal samples harbored two different *E. coli* clones, a total of 72 isolates were identified (see Table S2

in the supplemental material); of these a majority were *E. coli* (70/72, 97%) isolates, and there was one *Salmonella enterica* and one *Klebsiella pneumoniae* isolate. The fact that almost one of five dogs visiting this Parisian clinic was an ESBL/pAmpC carrier is alarming since pet ownership was significantly associated with ESBL *E. coli* colonization in humans (22). Hence, almost 20% of the owners considered here (including family members) could be at risk of acquiring these genes from their dogs. Moreover, in contrast to a recent report in diseased dogs in Germany (23), no carbapenemase producer was found. However, ChromID ESBL plates may have been impaired in detecting certain phenotypes, such as OXA-48 producers in the absence of ESBL coexpression.

This colonization rate is much higher than the one found in *E. coli* from diseased pets in Europe (7, 9) and in the United States (24). In healthy dogs, a higher ESBL prevalence has been reported only in China (25) or in studies more limited in size (11–13). Whether the situation in this clinic (among the most important ones throughout Paris) reflects the ESBL/pAmpC carriage rate in domestic dogs in the capital would need further study. Of note, owners were all different, and most ESBL/pAmpC *E. coli* isolates were clonally unrelated, as proved by PFGE (see Fig. S1 in the supplemental material). This argues for independent sources of ESBL/pAmpC producers and not for the spread of a limited number of clones. Consequently, this high colonization rate raises the question of a possible specific exposure to antibiotics of this dog subpopulation. Unfortunately, medical histories were not recorded. In any case, all dogs were healthy at sampling and were admitted for vaccination procedures, physical routine examination, or parasite screening. Further comparative studies with other canine clinics throughout Paris may help clarify this issue.

ESBLs were detected in 48 unrelated *Enterobacteriaceae* isolates (47 *E. coli* and 1 *K. pneumoniae*). Nineteen *E. coli* isolates belonged to phylogroup A, 12 belonged to B1, 13 belonged to D, and 3 belonged to B2, among which two belonged to the ST131 clone (one expressing a CTX-M-15 and one expressing a CTX-M group 9 enzyme). The ESBL phenotype was mostly due to the *bla*_{CTX-M-1}

TABLE 2 Plasmids associated with the *bla*_{ESBL} and *bla*_{CMY-2} genes in 70 *E. coli* isolates

Plasmid	pMLST/FAB ^a	No. of plasmids with the indicated phenotype/genotype	ESBL (<i>n</i> = 47)							Combination (AmpC, ESBL [<i>n</i> = 3])		
			AmpC CMY-2 (<i>n</i> = 20)	CTX-M group 9	CTX-M group 1			SHV-12	CMY-2, CTX-M-1	CMY-2, CTX-M-3	CMY-2, CTX-M group 9	
					CTX-M-1	CTX-M-15	CTX-M-32					
IncI1	ST2	16							1, 0	1, 0		
	ST3		2	22		1						
	ST5			1								
	ST7			1								
	ST13											
	ST16						1					
	ST27	1										
	ST29	1										
	ST36			1								
	ST133	1										
NT ^b	1			1								
IncF	F2:A-:B-		1		1							
	F18:A-:B-			1								
	F2:A2:B20		2									
	F46:A3:B1				1							
	F46:A-:B26		1									
NT					1		1					
HI1				2								
NT			1	2	1		2	0, 1	0, 1	1, 1		
Total		20	7	31	5	1	3	1	1	1		

^a Plasmid subtypes were classified according to their plasmidic MLST (pMLST) for IncI1 plasmids or to their FAB (FII, FIA, and FIB) formulas for IncF plasmids (19).

^b NT, not typeable.

gene (*n* = 31/45, 68.9%), while the *bla*_{CTX-M-15} (*n* = 6) and *bla*_{CTX-M-32} (*n* = 1) genes were rare. Seven *bla*_{CTX-M} group 9 and three *bla*_{SHV-12} genes were also identified. Interestingly, a 6% prevalence of fecal carriage of ESBL *E. coli* was observed in healthy subjects in Paris the same year and using the same methodology (26). The ST131 *E. coli* clone was dominant, yet in contrast to our study, results suggested limited commonality of ESBL *E. coli* clones between Parisian humans and dogs. However, CTX-M-1 was equally as abundant as CTX-M-15 in the human samples, which was a completely different proportion compared to clinical samples and which may indicate a nonhuman source of ESBLs. Whether dogs may have played a role in this ESBL epidemiology in humans, for instance, through contamination with transient *E. coli* transmitting CTX-M-1 plasmids to resident human *E. coli*, is an open question. Unfortunately, no information was given about the possible pet ownership of this human subpopulation.

Most *bla*_{CTX-M-1} genes were located on IncI1 plasmids (26/31, 83.4%), which surprisingly belonged mainly to the ST3 subtype (*n* = 22/26, 84.6%) (Table 2). The *bla*_{CTX-M-1}/IncI1/ST3 plasmid was previously recognized in several animal species and countries (6, 8, 10, 27, 28). However, such predominance was unexpected in this randomly tested dog population. This may underline a specific capability of this plasmid to persist in hosts and/or disseminate epidemically. The hypothesis that the veterinary clinic is the source of this plasmid is again unlikely since all animals had a very short stay in the clinic. As to a link with human carriage, CTX-M-1 plasmids were unfortunately not investigated from healthy Parisian subjects (26).

The nine remaining *bla*_{CTX-M-1} genes which did not belong to the IncI1/ST3 plasmid subtype were carried by four IncI1 plasmids, including rare subtypes (ST5, ST7, and ST36), two large (>450 kbp) IncHI1 plasmids, one IncF plasmid, and two nontypeable ones (Table 2). The unique *bla*_{CTX-M-3} gene was also on an IncI1/ST3 plasmid, whereas the six *bla*_{CTX-M-15} genes were on IncF (*n* = 4, including the *K. pneumoniae*), IncI1/ST16, or on a nontypeable (*n* = 1) plasmid. Four *bla*_{CTX-M-9} group genes were carried on IncF plasmids, including the widely detected F2:A-:B- plasmid; two others were located on an IncI1 plasmid (ST3 and nontypeable subtype), and the last one was on a nontypeable plasmid. Finally, the three *bla*_{SHV-12} genes were located on nontypeable plasmids. ESBL-producing *E. coli* presented multiple associated resistances (Table 1). Most of them were not present on ESBL plasmids since IncI1/ST3 plasmids that could conjugate (23 of the 25 IncI1/ST3 plasmids identified) were often resistant to tetracyclines and sulfonamides only (*n* = 12) or to the trimethoprim-sulfonamide combination (*n* = 7).

Cefoxitin resistance with a negative synergy test was detected in 21 isolates (20 *E. coli* and 1 *S. enterica*), which all presented the *bla*_{CMY-2} gene (Table 2). The emergence of this gene, which was pointed out in humans and animals in North and Central America in *S. enterica* and *E. coli* isolates (29), was also recently described in dogs, in the United Kingdom, the United States, and Greece (30–32). However, the proportion reported here (6.5%, ca. 30% of the isolates resistant to broad-spectrum cephalosporins) is surprisingly high. Phylogrouping showed that one *E. coli* isolate belonged to the A group, 5 belonged to B1, 13 belonged to D, and 1 belonged

to B2 (which was not ST131). Isolates were mainly not clonal even though identical PFGE profiles were identified in unrelated dogs (see Fig. S1B in the supplemental material).

The *bla*_{CMY-2} gene was systematically carried by a 90- to 100-kbp IncI1 plasmid. Most isolates (17/22 [77.2%]) consisting of 16 *E. coli* and 1 *S. enterica* presented a *bla*_{CMY-2}/IncI1/ST2 plasmid, whereas other subtypes were rare (one ST27 and one ST29) (Table 2). Even though IncA/C plasmids were reported as the main vehicles of the *bla*_{CMY-2} gene worldwide, IncI1 plasmids were also found, principally IncI1/ST12 or IncI1/ST23 (28, 32–34). In contrast, a high diversity of plasmids carrying *bla*_{CMY-2} was observed in American dogs (32). Interestingly, experimental treatment with cephalexin (a narrow-spectrum cephalosporin widely used for pets) proved to specifically select for the *bla*_{CMY-2}/IncI1/ST2 plasmid in unrelated dogs (35). Thus, our data unexpectedly suggest that this plasmid is also prevalent at a very high rate under field conditions, possibly reflecting the important use of cephalexin in dogs. Indeed, this efficient anti-staphylococcus molecule is not only prescribed as a first-line treatment in skin infections but also as preventive medication in noninfected surgeries. CMY-2-producing *E. coli* presented multiple associated resistances to doxycycline, fluoroquinolones, and sulfonamides, which are also widely used to treat pets in France. CMY-2-producing *E. coli* were also significantly more resistant than ESBL-producing *E. coli* to quinolones/fluoroquinolones or chloramphenicol (Table 1).

Finally, three isolates presented an ESBL/pAmpC association, expressing both a CMY-2 and a CTX-M enzyme (1 CTX-M-1, 1 CTX-M-3, and 1 CTX-M-9 group). Phylogrouping revealed three different types (A, B1, and D), and isolates were not clonal. Two *bla*_{CMY-2} genes were carried by an IncI1/ST2 plasmid, whereas the remaining *bla*_{CMY-2} plasmid and the three CTX-M plasmids were nontypeable.

In conclusion, our study reports a considerable ESBL/pAmpC reservoir in healthy dogs in Paris (18.5%) and a surprising distribution between the *bla*_{CTX-M-1}/IncI1/ST3 and *bla*_{CMY-2}/IncI1/ST2 plasmids among unrelated animals. Considering the prevalence of CTX-M-1 producers in healthy humans in Paris, the acquisition of CTX-M-1 plasmids from dogs is plausible. The transfer of CMY-2 producers to humans may also increase in the future. However, the reasons for the successful spread of these two specific plasmids remain unclear.

ACKNOWLEDGMENTS

This work was supported by the French Agency for Food, Environmental and Occupational Health and Safety.

We thank Claudine Tiberge for her skillful laboratory experience and Emilie Gay for her help with the statistical analyses.

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