Prevalence of Beta-Lactam and Quinolone/Fluoroquinolone Resistance in *Enterobacteriaceae* From Dogs in France and Spain—Characterization of ESBL/pAmpC Isolates, Genes, and Conjugative Plasmids

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Quantitative data on fecal shedding of antimicrobial-resistant bacteria are crucial to assess the risk of transmission from dogs to humans. Our first objective was to investigate the prevalence of quinolone/fluoroquinolone-resistant and beta-lactam-resistant *Enterobacteriaceae* in dogs in France and Spain. Due to the particular concern about possible transmission of extended-spectrum cephalosporin (ESC)-resistant isolates from dogs to their owners, we characterized the ESBL/pAmpC producers collected from dogs. Rectal swabs from 188 dogs, without signs of diarrhea and that had not received antimicrobials for 4 weeks before the study, were quantified for total and resistant *Enterobacteriaceae* on selective media alone or containing relevant antibiotic concentrations. Information that might explain antibiotic resistance was collected for each dog. Extended-spectrum cephalosporin-resistant isolates were subjected to bacterial species identification (API20E), genetic lineage characterization (MLST), ESBL/pAmpC genes identification (sequencing), and plasmid characterization (pMLST). Regarding beta-lactam resistance, amoxicillin- (AMX) and cefotaxime- (CTX) resistant *Enterobacteriaceae* and quinolone/fluoroquinolone-resistant, Nalidixic acid- (NAL) and ciprofloxacin- (CIP) resistant *Enterobacteriaceae* were detected in 70 and 18% of the dogs, respectively, whereas for quinolone/fluoroquinolone-resistance, Nalidixic acid- (NAL) and ciprofloxacin- (CIP) resistant *Enterobacteriaceae* were detected in 36 and 18% of the dogs, respectively. Medical rather than preventive consultation was a risk marker for the presence of NAL and CIP resistance. CTX resistance was mainly due to a combination of specific ESBL/pAmpC genes and particular conjugative plasmids already identified in human patients: $bla_{CTX-M-1}/IncI1/ST3 \ (n = 4)$, $bla_{CMY-2}/IncI1/ST12 \ (n = 2)$, and...
**INTRODUCTION**

Extended-spectrum and plasmidic-AmpC beta-lactamases (ESBL/pAmpC), which cause resistance to extended-spectrum cephalosporins (ESCs), are of considerable concern in veterinary and human medicine. This is because resistance to ESCs and co-resistance to other antimicrobial families (e.g., fluoroquinolones) limits the treatment options for infections with ESBL/pAmpC-producing bacteria. ESCs and fluoroquinolones, also used to treat animals, have been identified as critically important antibiotics in human medicine (1).

The emergence of ESBL/pAmpC-producing bacteria in animals and the environment is raising serious concerns (2, 3). In particular, the gastrointestinal tract of animals, including domestic pets, may serve as reservoirs of resistant bacteria that can cause opportunistic infections in vulnerable dogs and could be a source of contamination for humans. For example, dogs could possibly transmit ESC-resistant bacteria due to their close contacts with humans, the high consumption of beta-lactams in small animal veterinary practice (4), and the frequent occurrence of ESBL/pAmpC-producing *Escherichia coli* (5).

Recently, human-related pathogenic strains have been identified in dogs (6), and the sharing of identical ESBL/pAmpC between humans and dogs from the same households has also been demonstrated (7).

Although ESBL/pAmpC producers can spread clonally (8), there is evidence that mobile genetic elements carrying antimicrobial resistant genes can be transferred between bacteria, notably from commensal to pathogenic *Enterobacteriaceae* (9). Many studies have highlighted the existence of different *E. coli* bacteria harboring *blaCTX-M* or *blaCMY-2*-carrying plasmids, some being shared between human and animal strains (5). Such successful plasmids efficiently contribute to the spread of resistance determinants (10). In addition, the fact that many strains harboring *blaCTX-M-1* or *blaCMY-2* genes are resistant to other antimicrobial classes may also increase the threat. The use of expanded-spectrum cephalosporins and of fluoroquinolones in dogs is known to select ESBL/pAmpC producers in the fecal microbiota (11–13).

Over the past 5 years, the presence of ESBL/pAmpC genes in *Enterobacteriaceae* strains from the feces of healthy dogs in Europe has been reported in several studies (5, 7, 14–16). However, information on the clonality of the ESBL/pAmpC isolates and knowledge about the plasmids carrying these ESBL/pAmpC genes is much more limited (5, 15, 17, 18).

Thus, examination of the occurrence of ESBL/pAmpC bacteria together with plasmids circulating in healthy pets, and their associated co-resistance, is of major importance with respect to the risk of transfer to humans in daily situations of close contact and will also extend understanding of the dissemination and persistence of ESBL and pAmpC beta-lactamase genes.

The present study was designed to assess the prevalence of beta-lactam and quinolone/fluoroquinolone resistance in fecal *Enterobacteriaceae* from dogs in Spain (Madrid) and France (Toulouse), to identify the potential risk factors for the presence of antibiotic resistance, and to characterize the ESC-resistant isolates, the genes coding for and plasmids carrying ESBL/pAmpC resistance in those isolates.

**MATERIALS AND METHODS**

**Isolation of *Enterobacteriaceae* Strains**

Between March 2013 and January 2014, fecal specimens from 269 different dogs presented at two veterinary clinics (158 at the small animal clinics of the veterinary teaching hospital in Toulouse, France and 111 at a veterinary clinic in Madrid, Spain) were sampled. These dogs were being admitted for a preventive health service, vaccination or medical consultation (disease, trauma, etc.). Dogs were included in the study if they had not received any systemic or local antimicrobial during the previous 4 weeks, according to the owner. Animals with diarrhea were not included in the study. Breed, age, weight, diet (commercial, homemade, other), and reason for the visit (preventive, medical) were recorded for each dog. According to EU law (Directive 2010/63/UE), procedures which use animals according to common veterinary practice did not require minister permission and ethics committee opinion. Sampling was performed by veterinarians in veterinary clinics. They consisted in non-invasive samples (rectal swabbing) performed on client-owned animals and were acquired with the written consent from all owners.

Samples were collected by swabbing, immediately suspended in liquid amies preservation medium (Copan eSwabs Amies liquid, Biomerieux), stored at +4°C and analyzed within 24 h after sampling. Ten-fold dilutions were prepared in 0.9% sterile saline. One hundred microliter were inoculated (i) on MacConkey agar not supplemented with antimicrobials to obtain counts of total *Enterobacteriaceae*; (ii) on MacConkey agar supplemented with amoxicillin (AMX 100 µg/mL) or cefotaxime (CTX 1.5 µg/mL) to obtain counts of penicillin- or fluoroquinolones also used to treat animals, have been identified for infections with ESBL/pAmpC-producing bacteria. ESCs and extended-spectrum cephalosporins (ESCs), are of considerable importance in human medicine (1).

ESBL/pAmpC plasmids were located in different genetic lineages of *E. coli*, with the exception of two strains in France (ST6998) and two in Spain (ST602). Our study highlights dogs as a potential source of Q/FQ-resistant and ESBL/pAmpC-producing bacteria that might further disseminate to humans, and notably a serious risk of future acquisition of CTX-M-1 and CMY-2 plasmids by the owners of dogs.
cefotaxime-resistant Enterobacteriaceae, and (iii) on MacConkey agar supplemented with nalidixic acid (NAL 20 μg/mL) or ciprofloxacin (CIP 2 μg/mL) to obtain counts of quinolones- (Q) or fluoroquinolones- (FQ) resistant Enterobacteriaceae. These antibiotic concentrations have been chosen according to the European Committee on Antimicrobial Susceptibility Testing Clinical breakpoint (www.eucast.org) to obtain non-susceptible/resistant Enterobacteriaceae.

All batches of media supplemented with antibiotics were tested with positive (resistant) and negative (susceptible) control strains. Total or resistant Enterobacteriaceae (cfu/mL) were counted after incubating these MacConkey agar plates for 24 h at 37°C. The limit of detection was 10 cfu/mL of undiluted liquid preservative medium. The prevalence of dogs carrying resistant Enterobacteriaceae (%) was estimated. Samples containing at least 10⁴ total Enterobacteriaceae/mL were included in the analysis to limit the risk of underestimating the prevalence. Of the 269 fecal swabs collected, 188 (n = 90 from Toulouse and n = 98 from Madrid) were included in the analysis.

Multivariate logistic regression analyses were performed to identify independent predictors (sampling site, age, weight, diet, and reason for visit) of resistance carriage (prevalence of AMX-R, CTX-R, NAL-R, and CIP-R) among the investigated animals, using Systat v13.1 version. Statistical significance was set at p < 0.05. Comparisons of resistance abundance were done using a chi-square test with the significance level set at 0.05.

Identification and Typing of CTX-Resistant Bacteria

CTX-resistant colonies of Enterobacteriaceae were collected (one colony per animal), and identified by using API 20E galleries (bioMérieux). Non-Enterobacteriaceae isolates (mostly Pseudomonas spp.) were discarded.

Multilocus sequence typing (MLST) was carried out according to the protocol described on the E. coli (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and C. freundii (https://pubmlst.org/cfreundii) MLST website.

Antibiotic Susceptibility Testing

The antibiotic susceptibilities of CTX-R E. coli and their transconjugants were determined by disk diffusion method according to CLSI protocols (19). The following antibiotic disks were used: ampicillin (10 μg), amoxicillin (20 μg) plus clavulanic acid (10 μg), cefoxitin (30 μg), ceftizime (30 μg), cefotaxime (30 μg), cephepine (30 μg), aztreonam (30 μg), imipenem (10 μg), streptomycin (10 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), tetracycline (30 μg), chloramphenicol (30 μg), trimethoprim (5 μg), and sulfonamides (300 μg) (Bio-Rad, Marnes-la-Coquette, France if available or Oxoid, Dardilly, France). The susceptibility breakpoints for all antimicrobials were those recommended by CLSI (20).

ESBL production was detected by double-disk synergy test on Mueller-Hinton agar between clavulanic acid and ceftazidime, cefotaxime, or cefepime (20, 21). AmpC beta-lactamases detection was based on the inhibitory effect of cloxacinil on AmpC production observed on plates supplemented with 200 mg/L cloxacinil. The control strains used were E. coli ATCC 25922, K. pneumoniae ATCC 700603, and K. pneumoniae CMY-2 from Pr R. Bonnet, France.

Characterization of blaESBL/AmpC Genes

After bacterial DNA extraction with a DNeasy blood and tissue kit (Qiagen, Hilden, Germany), the blaTEM, blaSHV, blaCTX-M, blaCMY-2, genes were detected by PCR and sequenced using specific previously-described primers (22, 23). Sequences were analyzed by the BLAST Internet services (www.ncbi.nlm.nih.gov/BLAST).

Transferability of the blaESBL/pAmpC Genes and Plasmid Characterization

Plasmids were transferred by conjugation to rifampin-resistant E. coli recipient strains. One-milliliter of a LB culture of the ESBL/AmpC-producing donor strain (10⁸ cfu/mL) and one mL of the J0C0J7-rifR4 rifampicin-resistant recipient (10⁸ cfu/mL) strain were added to 3 mL LB broth and incubated for 2 h at 37°C without agitation. Transconjugants were selected on LB agar containing 1.5 μg/mL of cefotaxime and 100 μg/mL of rifampicin. Plasmid DNA from transconjugants was purified and resolved by electrophoresis in 0.7% agarose, as described previously (24), to confirm that cells carried only one conjugative plasmid.

Plasmid replicon types were determined using the PCR-based replicon typing (PBRT) scheme (25) and plasmid sequence subtypes by applying plasmid multilocus sequence typing (pMLST) for IncI1 (http://pubmlst.org/plasmid/).

RESULTS

Prevalence of Resistant Enterobacteriaceae

The characteristics of the dog population studied are given in Table 1. A total of 188 dogs (90 from Toulouse and 98 from Madrid), with ages ranging from 4 months to 16 years (median age 5 years), and feces containing at least 10⁴ total Enterobacteriaceae, were included in the study. Body weights ranged from 1 to 53 kg (median weight 10 kg), and most of the dogs (73%) were fed with commercial feed. Eighty-four dogs (44.5%) were admitted for medical consultation, mainly in Toulouse (36.2%), most of them for skin disorders, whereas 94 dogs (50.0%) were admitted for preventive consultation, mainly in Madrid (38.8%), the majority being admitted for vaccination.

The prevalences of dogs carrying beta-lactam- or Q/FQ-resistant bacteria and its association with predictor (explanatory) variables are shown in Table 2 and Figure 1. Regarding the beta-lactam-resistant Enterobacteriaceae in feces, approximately 70% of the dogs carried AMX-R and 18% carried CTX-R Enterobacteriaceae (19% in Toulouse and 16% in Madrid). Multivariate regression analysis revealed that dogs in Toulouse were more frequently colonized by AMX-R Enterobacteriaceae (78%) than those from Madrid (63%; p < 0.01). The reason for consultation, diet, age and weight were not predictors of beta-lactam resistance carriage among animals (Table 2 and Figure 1).
TABLE 1 | Distribution of the predictor variable from dogs in Madrid and Toulouse veterinary clinics.

| Risk marker            | Madrid N (%) | Toulouse N (%) | Total N (%) |
|------------------------|--------------|----------------|-------------|
| Reason for consultation| Medical 16 (9) | 68 (36)        | 84 (45)     |
|                        | Preventive 73 (39) | 21 (11)        | 94 (50)     |
|                        | Unknown 9 (5) | 1 (1)          | 10 (5)      |
| Diet                   | Commercial 76 (40) | 61 (32)        | 137 (73)    |
|                        | Home 4 (2) | 4 (2)          | 8 (4)       |
|                        | Mixed/other 10 (6) | 6 (3)          | 16 (9)      |
|                        | Unknown 8 (4) | 19 (10)        | 27 (14)     |
| Age (year)             | ≤5 54 (29) | 48 (26)        | 102 (54)    |
|                        | >5 42 (22) | 39 (21)        | 81 (43)     |
|                        | Unknown 2 (1) | 3 (2)          | 5 (3)       |
| Sex                    | Female 50 (27) |               |             |
|                        | Male 39 (21) |               |             |
|                        | Unknown 98 (52) | 1 (1)          |             |
| Weight (Kg)            | >20 17 (9) | 37 (20)        | 54 (29)     |
|                        | >10–20 13 (7) | 16 (9)         | 29 (15)     |
|                        | ≤10 66 (35) | 33 (18)        | 99 (53)     |
|                        | Unknown 2 (1) | 4 (2)          | 6 (3)       |

Regarding quinolone/fluoroquinolone-resistant Enterobacteriaceae, the overall frequency of carriage of NAL-R bacteria was 36% (38% in Toulouse and 35% in Madrid) and that of CIP-R bacteria was 18% (20% in Toulouse and 15% in Madrid). The multivariate logistic regression analysis indicated that the reason for the dog’s visit to the veterinary clinic was a predictor of Q/FQ resistance \( (p < 0.05) \). Animals brought in for preventive measures were less likely to carry Enterobacteriaceae resistant to Q/FQ than those presented for medical treatment \( (27 \text{ vs. } 45\% \text{ for NAL and } 12 \text{ vs. } 25\% \text{ for CIP}) \). Dog weight was also found to be a predictor of resistance prevalence, but only for NAL \( (p < 0.01) \). The population of dogs harboring fecal bacteria resistant to NAL weighed more (median = 14.30 kg) than the population of dogs without resistant bacteria (median = 9.10 kg). A similar trend, but without statistical significance, was apparent for other antibiotics (CTX and CIP).

Characterization of ESBL/AmpC Producers, Genes, Plasmids

ESBL/AmpC genes and the plasmids carrying them were identified in 14 cefotaxime-R Enterobacteriaceae isolated from the feces (Table 3). They included 10 ESBL-producing E. coli and 4 AmpC-carrying strains \( (2 \text{ E. coli, } 1 \text{ P. mirabilis having acquired AmpC enzyme and } 1 \text{ C. freundii that carries naturally a chromosomal AmpC}) \). According to the MLST database, the 12 E. coli were associated with 10 different sequence types (STs) of which nine have been previously reported. ESBL-producing E. coli presented multiple associated resistances. All strains were resistant to sulfonamides and tetracycline, and some to trimethoprim (5/10), chloramphenicol (4/10), and streptomycin (2/10). ESBL-producers in Madrid were always co-resistant to...
NAL and CIP whereas those isolated in Toulouse were not, despite the presence of a significant level of Q/FQ resistant strains in the feces. The ESBL phenotype was mostly due to the bla\textsubscript{CTX-M-1} gene (n = 6), whereas the bla\textsubscript{CTX-M-15} gene (n = 1 in Toulouse) was rare. The 6 bla\textsubscript{CTX-M-1} genes were systematically carried by the conjugative IncI1/ST3 plasmid subtype that conferred additional resistance to tetracyclines and sulphonamide. The bla\textsubscript{CTX-M-1} IncI1/ST3 plasmid was found in isolates belonging to four different STs (ST6998 (n = 2), ST227 (n = 1), and ST38 (n = 1) in Toulouse and ST602 (n = 2) in Madrid). The bla\textsubscript{CTX-M-15} gene was on a conjugative IncI1/ST31 plasmid which did not confer other resistances to its host strain.
which belonged to ST2449. Three \textit{bla}SHV−12 genes were also identified, only in Madrid, and were carried by a diversity of conjugative plasmids found in MLST clonally unrelated \textit{E. coli}: \textit{bla}SHV−12 IncI1/ST3 plasmid associated with ST453(STC86), \textit{bla}SHV−12 IncI1/ST26 with a new ST and \textit{bla}SHV−12 IncFII plasmid with ST1594.

AmpC beta-lactamases were detected in 4 isolates: 1 \textit{C. freundii} isolated from Toulouse dogs and 2 \textit{E. coli}, 1 \textit{P. mirabilis} from Madrid. As CMY-2 is the most common type of AmpC and \textit{pAmpC} enzymes among \textit{Enterobacteriaceae}, we have focused on the detection of \textit{bla}CMY−2 gene. For the 2 \textit{E. coli}, belonging to ST10 and ST12, the \textit{bla}CMY−2 gene was carried by the IncI1/ST12 conjugative plasmid, one carrying only streptomycin resistance while the second was associated with multiple resistances such as sulfonamide, tetracycline, streptomycin and chloramphenicol. The remaining 2, the \textit{bla}CMY−2 gene in \textit{P. mirabilis} and the \textit{bla}CMY−80 gene in \textit{C. freundii}, were not located on conjugative plasmids.

**DISCUSSION**

The resistance phenotypes investigated in this study are of high clinical relevance in small animal veterinary practice in view of the widespread use of beta-lactams and fluoroquinolones for treating common infections in dogs (4) and the potential risk of resistance transmission (bacteria or genes) to humans through direct contact (1).

**Prevalence of Resistant \textit{Enterobacteriaceae}**

Even if comparison between different studies is difficult due to biases associated with methodological factors, the high prevalences of AMX-R (70%) and CTX-R (18%) \textit{Enterobacteriaceae} were similar to those already reported, over the last 5 years, in healthy dogs in various European countries (5, 14, 26, 27), but higher than those reported in Denmark, Sweden and the UK (7, 15, 28). In our study, the prevalence of AMX-R isolates included \textit{Enterobacteriaceae} species naturally resistant to penicillin like \textit{K. pneumoniae}, \textit{C. freundii}, \textit{Enterobacter} and those having acquired such resistance like \textit{E. coli}, \textit{P. mirabilis}.

Similarly, a high prevalence of NAL-R (35–38%) and CIP-R (15–20%) carriers was detected, as previously reported in Portugal (27), but higher than that reported in the UK (29). The use of critically important antimicrobials (3rd and 4th generation cephalosporin and quinolone/fluoroquinolone) is more commonly cited as being prescribed for dogs in Spain, France and Germany than in Sweden and the UK (4). We therefore believe that the observed high prevalence of cefotaxime (CTX) and \textit{Q/FQ} resistance in dogs may be associated with the use of critically important antimicrobials for canine treatments.

The prevalence of AMX-R was higher in Toulouse (78%) than in Madrid (63%) but such result is difficult to interpret in light of antibiotic usage data described in the literature for the two countries (4). Dog weight (Kg) was a risk marker significantly associated with nalidixic acid resistance whereas age was not.
This result is difficult to explain but the possible difficulty of precisely administering the recommended dose according to the dog’s weight might sometimes lead to over-dosing (over-exposure of fecal flora to antibiotics) or under-dosing (which might necessitate a change of antibiotic), both situations promoting the development of antibiotic resistance.

The significantly higher prevalence of NAL-R and CIP-R in dogs presented for medical consultation has already been reported (27) and might be related to the fact that healthy animals have fewer opportunities for contact with antimicrobials. However, the past medical histories of these dogs were not recorded. Exposure to quinolones had previously been indicated as a risk factor for the emergence of resistance to several antibiotics in E. coli isolated from dogs (27) but the involvement of other antimicrobial classes (e.g., tetracyclines, sulfonamides, aminoglycosides), that are frequently used in canine therapy, cannot be excluded. Because no antimicrobial was administered to the dogs during the month prior to sampling, our results are consistent with the hypothesis that the reversibility of resistance in the absence of antimicrobials is a slow process.

It is noteworthy that as almost 20% of the dogs, independently of origin, were CTX-R (ESBL or AmpC) or FQ-R carriers, the owners (including family members) could be at risk of acquiring these resistant bacteria and genes from their dogs (7) especially if they are carried by conjugative plasmids. FQ resistance was mainly mediated by mutation in chromosomal genes whereas plasmid-mediated FQ resistance (PMQR) determinants only confer low-level resistance to fluoroquinolones. On the 16 CIP-R and 27 NAL-R Enterobacteriaceae isolated in our study, we performed the detection for predominant PMQR qnrA, qnrB, qnrS, and aac(6’)-Ib-cr by PCR as described elsewhere (30, 31). No PMQR were detected (data not shown) but we know that among fluoroquinolone non-wild Enterobacteriaceae, only 20% carried at least one PMQR determinant (32).

**Characterization of ESBL/AmpC Producers, Genes, and Plasmids**

Among the ESBL genes identified in Enterobacteriaceae isolated from different dogs in the two countries, the blaCTX−M−1 gene was exclusively located on the specific conjugative IncI1/ST3 plasmid. Although this study is the first to identify blaCTX−M−1 on IncI1/ST3 plasmid in Spain, such a combination has recently been reported in France in healthy urban dogs (5), in both diseased and healthy humans (33) but also in food-producing animals and the environment (22, 34). The blaCTX−M−1 on IncI1/ST3 plasmid was detected in four different E. coli ST, including ST38 and ST602 which have frequently been identified in different situations (human pathogens or not, livestock, environment) but are only rarely found in dogs. The E. coli ST38 and ST602 lineages carrying blaCTX−M−1 on IncI1/ST3 plasmids have previously been reported in food-producing animals in Portugal (35) and healthy dogs (36) in Tunisia, respectively. The diversity of the analyzed strains (date and place of isolation) and the diversity of the E. coli ST, in contrast to the homogeneity of the blaCTX−M−1 plasmid type, confirm the specific capability of the blaCTX−M−1-carrying IncI1/ST3 plasmid to disseminate through unrelated bacterial strains.

In our study, one blaCTX−M−15/IncI1/ST31 conjugative plasmid, carrying an additional blaTEM−1 gene without additional resistance, was identified in a French isolate. Such a plasmid has already been detected in E. coli and Shigella sonnei clinical isolates from cattle and humans in different European countries (37, 38). This plasmid was found in E. coli ST2449 which has been reported once for a human pathogen in Europe and twice on the American continent for animal pathogens (http://enterobase.warwick.ac.uk/species/ecoli/search_strains).

Three blaSHV−12 genes were identified only in Spain and were carried by three different conjugative plasmids: the blaSHV−12 IncI1/ST3 plasmid previously reported in 2 E. coli of poultry origin in Italy (39), the blaSHV−12 IncI1/ST26 plasmid previously identified in E. coli of human origin in Italy (39), from food animals and products in Portugal (35) and the blaSHV−12 F76:A:B-FII plasmid not yet described. In addition, SHV-12-carrying plasmids were located in three different genetic lineage strains. Among them, a new ST and 2 other STs (ST453, ST1594) that have occasionally been detected in livestock and poultry on different continents as well as in the environment and humans for ST453 (40, 41). The ability of blaSHV−12 genes to combine with different plasmids allows the enzymes to attain diverse niches worldwide, as previously demonstrated (42). Prevalence of the blaSHV−12 gene seemed higher in Spain but the number of cefotaxime-R isolates in France was too small to draw a conclusion.

Three AmpC were detected among the eight cefotaxime-R Enterobacteriaceae in Spain but only one in France. The blaCMY−2 on IncI1/ST12 plasmid was the only conjugative plasmid-encoded pAmpC gene that we detected in two different E. coli lineages (ST10 and ST12). The E. coli ST10 and ST12 have repeatedly been isolated from various reservoirs i.e., healthy or diseased animals including dogs, humans and the environment (40). Previous studies support the hypothesis that plasmids play the major role in the transmission of blaCMY−2 between reservoirs. The widespread distribution of blaCMY−2-carrying IncI1/ST12 plasmid has been reported among various E. coli STs from human patients, livestock animals, food, environment, and also from healthy dogs in Tunisia (17, 35, 36, 41, 43). However, to our knowledge, this is the first time that the blaCMY−2 IncI1/ST12 plasmid has been identified in healthy dogs in Europe although it was recently identified in a single clinical isolate associated with another ST in Denmark (17).

Until now, the blaCMY−2 on IncI1/ST2 plasmid was the only one reported in healthy dogs in Europe (5, 11, 17). E. coli ST10 harboring blaCMY−2 on the IncI1/ST12 plasmid seems to circulate in European broiler production (17). In addition to food animals, which are often proposed as possible reservoirs for blaCMY−2 in humans through the spread of specific plasmids, our results also raise questions about the possible contribution, to this burden in Europe, of blaCMY−2-carrying IncI1/ST12 plasmid from companion animals.

The third AmpC-carrying isolate in Spain was a CMY-2-producing Proteus mirabilis in which mobilization of the
$bla_{CMY-2}$ gene does not involve a plasmid. Hybridization experiments are needed to confirm the chromosomal location of this $bla_{CMY-2}$ gene. In France, a single CMY-80-producing Citrobacter freundii was found in canine feces and the $bla_{CMY}$ variant gene was not carried by a conjugative plasmid. Such a $bla_{CMY}$ variant was only found in C. freundii isolated from human urine samples in Spain (GenBank Accession Number JQ733577).

Overall, a diversity of $bla_{ESBL/pAmpC}$ genes were found but the $bla_{CTX-M-15}$ which is highly predominant in the ESBL-producers in human and dog pathogens seems minor in the commensal digestive flora (44–47). In the other hand, $bla_{CTX-M-1}$ and $bla_{SHV-12}$ have recently emerged in dog pathogens (48). ESBL/AmpC genes were found in different clonal lineages of E. coli but not in the most prevalent ST in human (ST131, ST648) and dog (ST410) pathogens. However, some successful ESBL/pAmpC plasmid lineages have been described in human and dog pathogens.

In our study, most of the ESBL/AmpC strain isolated from Madrid presented the broadest multiresistance pattern, showing co-resistance to ESC and FQ. Similarly, such co-resistance in E. coli from food or food-producing animals in Europe was shown to occur at the highest frequency in Spain (48). We performed the detection for predominant PMQR $qnrA$, $qnrB$, $qnrS$, and $aac(6’)-Ib-cr$ by PCR as described elsewhere on ESBL/AmpC strain but no PMQR were detected except a $qnrA$ gene in the AmpC-producer C. freundii (data not shown). In our study, the low prevalence of PMQR contrast with the high prevalence previously described in dog pathogens (32, 44, 49). Anyway, multiresistance to different classes of antibiotic used in veterinary medicine, conferred by conjugative plasmids and/or by the host strains, may increase the potential risk of co-selection, maintenance, transmission, and propagation of the multidrug-resistant E. coli isolates and/or multidrug-resistant plasmids in digestive tract. Moreover, despite the few strains studied here, and in addition to the greater multiresistance reported in Spain, our results suggest that the diversity of ESBL/pAmpC genes and plasmids is greater in Spain than in France. This may be due to a geographical factor, differences in the use of antibiotics in dogs or differences in the distribution of ESBL/AmpC strains and plasmids in the environment of dogs (humans, water, and food).

The sharing of ESBL/pAmpC-carrying plasmids between healthy dogs and humans is of particular concern.

In conclusion, our study reveals a high prevalence of resistance to fluoroquinolones (18%) and extended-spectrum-cephalosporins (18%) in feces from dogs in Toulouse and Madrid. The state of health of the animals is a risk factor for quinolone-resistance carriage, probably related to previous treatments. In unrelated dogs in Spain and/or France, the spread of extended-spectrum-cephalosporinases can be due to successful combination of particular ESBL/pAmpC genes and specific plasmid ST, as for $bla_{CTX-M-1}/IncI1/ST3$ and $bla_{CMY-2}/IncI1/ST12$. On the contrary, the $bla_{SHV-12}$ gene combines with different plasmid lineages. Such highly conjugative plasmids have previously been described in healthy or pathogenic E. coli isolates of human, animal and food origin. They are located in different E. coli ST, and some are increasingly being identified in various known reservoirs. Considering the prevalence of resistance to fluoroquinolones and extended-spectrum-cephalosporinases (notably the large reservoir of CTX-M-1 and CMY-2 producers in dogs) there is a serious and plausible risk of future acquisition of CTX-M-1 and CMY-2 plasmids by their owners.

DATA AVAILABILITY

The datasets generated for this study are available on request. The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

VD, MA, SS-J, NS-L, BG-Z, AA, and AB-M designed the study, M-CC and DL-P collected the feces. VD, MA, GM, DB, and NA performed bacterial counts and the storage of resistant isolates. VD carried out the statistical tests and analyzed the DNA sequences. VD and NA analyzed the ESBL/AmpC isolates. VD, JdG, BG-Z, AA, and AB-M drafted the manuscript. All the authors edited the manuscript and approved the paper.

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