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Molecular characterization of quinolone resistance mechanisms and extended-spectrum β-lactamase production in *Escherichia coli* isolated from dogs

D. Meireles[a,b], L. Leite-Martins[a,c], L.J. Bessa[a,d], S. Cunha[a,b], R. Fernandes[b], A. de Matos[c,e], C.M. Manaia[f], P. Martins da Costa[a,d,*]

[a] *Produção Aquática, ICBAS – Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal*
[b] *Ciências Químicas e das Biomoléculas, Escola Superior de Tecnologia de Saúde do Porto, Instituto Politécnico do Porto, Porto, Portugal*
[c] *Clínica Veterinária, ICBAS, Universidade do Porto, Porto, Portugal*
[d] *CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Porto, Portugal*
[e] *CECA – Centro de Estudos de Ciência Animal, ICETA – Instituto de Ciências e Tecnologias Agrárias e Agro-Alimentares, Universidade do Porto, Porto, Portugal*
[f] *CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal*

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ABSTRACT

The increasing prevalence of antimicrobial resistances is now a worldwide problem. Investigating the mechanisms by which pets harboring resistant strains may receive and/or transfer resistance determinants is essential to better understanding how owners and pets can interact safely. Here, we characterized the genetic determinants conferring resistance to β-lactams and quinolones in 38 multidrug-resistant *Escherichia coli* isolated from fecal samples of dogs, through PCR and sequencing. The most frequent genotype included the β-lactamase groups TEM ($n=5$), and both TEM + CTX-M-1 ($n=5$). Within the CTX-M group, we identified the genes CTX-M-32, CTX-M-1, CTX-M-15, CTX-M-55/79, CTX-M-14 and CTX-M-2/44. Thirty isolates resistant to ciprofloxacin presented two mutations in the *gyrA* gene and one or two mutations in the *parC* gene. A mutation in *gyrA* (reported here for the first time), due to a transversion and transition (TCG → GTG) originating a substitution of a serine by a valine in position 83 was also detected. The plasmid-encoded quinolone resistance gene, *qnrs1*, was detected in three isolates. Dogs can be a reservoir of genetic determinants conferring antimicrobial resistance and thus may play an important role in the spread of antimicrobial resistance to humans and other co-habitant animals.

1. Introduction

The emergence of antimicrobial resistance (AMR) in animals is of major public health significance owing to the risk of bacteria and resistance genes these animals carry may spread to humans [1]. Over the last decades, improvements in medical assistance have enabled pets to be treated in veterinary hospitals and subjected to antibiotic therapies very similar to the ones used in human medicine [2]. This reality coupled with the intimate contact that exists between pets and their owners and negligence in hygiene practices by humans [3], makes companion animals important players in the global dissemination of AMR. Even so, there still is a lack of information on the transfer of antimicrobial resistant strains from pets to humans and vice versa. As a result, directing investigations focused on the analysis of microorganisms isolated from pets and the mechanisms by which they may receive and/or transfer resistance determinants, as well as implementing AMR surveillance, is essential to designing new strategies and interventions aimed at preventing the dissemination of resistance and enabling safer interactions between humans and pets.

Escherichia coli has been used as a reference species to track the spread of AMR. Besides being a genetic versatile commensal of both humans and animals, it is also widely disseminated in the environment (water, soil and food) [4] and can constitute an important reservoir of antibiotic resistance determinants that can be readily transferred to and from other pathogenic bacteria [5]. The production of extended-spectrum β-lactamases (ESBLs) and the development of resistance to quinolones are common in *E. coli*

* Corresponding author at: Produção Aquática, ICBAS – Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal. Tel.: +351 220428315.
E-mail address: pmcosta@icbas.up.pt (P. Martins da Costa).

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strains and represent a serious threat to both human and animal health. Most of the current scientific literature reporting the presence of ESBL-producing *E. coli* in animals has focused on production animals (cattle, swine, rabbits) and wild animals (gulls and birds of prey) [6,7]. However, recent corresponding studies focusing on companion animals have started to be performed [8–13]. Since dogs and cats are kept in close contact with their owners, silent within-household transmission of resistant bacteria and resistance genes may occur in either direction by direct contact or, indirectly, through household surfaces and objects contaminated by feces, oral secretions or urine [3,14,15].

Mutations on the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* genes are the main cause of quinolone resistance in *E. coli*. It has been suggested that plasmid-mediated quinolone resistance (PMQR) due to *qnr*, qepA and aac(6′)-Ib-cr genes may facilitate the selection of chromosomal encoded quinolone resistance mechanisms [16] as well as other multidrug resistance phenotypes [17].

Here, we characterize the presence of resistance determinants to two classes of antimicrobials (β-lactams and quinolones) in 38 multidrug-resistant *E. coli* isolated from fecal samples collected from randomly selected dogs attending the Veterinary Hospital of the University of Porto (UPVet), in Porto, Portugal. We focused on investigating the diversity of resistance determinants and the co-occurrence of ESBL and PMQR genes or mutations in the QRDR of the *gyrA* and *parC* genes.

2. Materials and methods

2.1. Sample collection and E. coli isolation

Between September 2009 and May 2012, 78 dogs attending the Veterinary Hospital of the University of Porto (UPVet), either for a normal checkup or due to disease, were sampled in order to detect the presence of drug-resistant *E. coli* in their feces. To be eligible for sampling, animals must not have received antimicrobial therapy in the last four months. Approval was obtained from the Ethics Committee of the Abel Salazar Institute for the Biomedical Sciences, University of Porto. Fecal samples were collected and immediately diluted (1:10) in a saline buffer and stored at room temperature for 30 min. From the initial suspension, an aliquot of 5 μl was streaked on Tryptone Bile X-glucuronide agar (TBX; Biokar Diagnostics, Allonne, Beauvais, France) and 100 μl was spread on the same culture medium containing 2 μg/ml of cefotaxime. The plates were incubated at 37 °C for 24 h. Two to five well-defined colonies with the typical appearance of *E. coli* were selected from each non-supplemented TBX agar plate and, additionally, all colonies presenting different morphologies were picked from the cefotaxime supplemented TBX agar plates. Standard biochemical methods were used for the confirmation of *E. coli* isolates, as described elsewhere [7]. The isolates were stored in culture medium supplemented with 20% glycerol at −20 °C. A total of 307 *E. coli* isolates were recovered from the fecal samples collected from dogs ($n = 78$).

2.2. Antimicrobial susceptibility characterization

Resistance patterns were determined by the agar disk-diffusion method on Mueller–Hinton agar (BioKar Diagnostics) according to the CLSI guidelines [18]. The antimicrobial agents (Oxoid Basingstoke, UK) that we used included ampicillin (AMP, 10 μg), amoxicillin–clavulanic acid (AMC, 30 μg), aztreonam (ATM, 30 μg), cephalothin (CEF, 30 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), cefoxitin (FOX, 30 μg), imipenem (IPM, 10 μg), gentamicin (GEN, 10 μg), amikacin (AMK, 30 μg), streptomycin (STR, 10 μg), tobramycin (TOB, 10 μg), kanamycin (KAN, 30 μg), ciprofloxacin (CIP, 5 μg), nalidixic acid (NAL, 30 μg), tetracycline (TET, 30 μg), chloramphenicol (CHL, 30 μg), trimethoprim–sulfamethoxazol (SXT, 25 μg) and nitrofurantoin (NIT, 300 μg). The ESBL phenotype in *E. coli* culture was observed on plate according to the method of disk approximation [19]. Multidrug resistance was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories [20]. The minimum inhibitory concentration (MIC) of CIP was determined by the broth microdilution method [18] for the isolates that had shown more uncommon genotypes conferring quinolone resistance.

2.3. E. coli selection for further studies

For further molecular characterization (phylogenetic group determination, detection of β-lactams and quinolones resistance genes), a total of 38 multidrug-resistant *E. coli* with ESBL and/or quinolone resistance phenotypes (Table 1) were selected from the overall strains ($n = 307$) recovered from fecal samples of randomly selected dogs ($n = 78$). To avoid clone duplication, only one isolate among all isolates from the same sample presenting identical AMR profiles was selected.

2.4. E. coli phylogenetic group determination

DNA was extracted through cell lysis by boiling in the presence of the Instagene Matrix (BioRad Laboratories, California, USA) as described by the manufacturer. A multiplex polymerase chain reaction (PCR) was performed for phylogenetic group (A, B1, B2 or D) determination, as previously described [21]. The PCR products were analyzed using electrophoresis in 1.5% agarose gel (Seakem Agarose, Maine, USA), at 150 V for 45 min. The gels were stained with ethidium bromide (0.5 μg/ml) and photographed under ultraviolet light using a Molecular Imager Gel Doc XR (BioRad Laboratories). Positive controls were used in the PCR analysis.

2.5. Characterization of resistance genes

The presence of β-lactamase genes as well as the presence of mutations in the QRDRs of *gyrA* and *parC* genes and of *qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, *qepA* and *aac(6′)-Ib-cr* genes, herein referred as PMQR genes, were screened using PCRs according to the protocols of Dallenne and coworkers [22] and Figueira et al. [23], respectively. Positive control strains were used in each set of PCRs, as described in previous studies [24,25]. For DNA Sanger sequencing (Macrogen, Amsterdam, The Netherlands), PCR products were purified using the GRS PCR & Gel Band Purification Kit (Grisp, Porto, Portugal). The mutations in the *gyrA* and *parC* genes were detected after analysis of the nucleotide sequences, as described before [23]. Nucleotide sequences of the amplicons of the *gyrA* and *parC* genes were aligned using Clustal W in MEGA 5.10 software. We made a comparison with the homologous nucleotide sequences of quinolone-susceptible wild-type *E. coli* K-12. In the case of β-lactamase genes, only the $bla_{CTX\text{-}M}$ genes were sequenced. Each sequence was then compared with already known sequences of the respective gene by multiple-sequence alignment using BLAST.

3. Results

3.1. Antimicrobial resistance phenotypes and phylogenetic group determination

Among the 307 *E. coli* isolates, a set of 38 multidrug-resistant strains exhibiting ESBL phenotypes and/or quinolone resistance was chosen for further genetic analysis that allowed the detection

Table 1
Characteristics of 38 multidrug-resistant *E. coli* isolates collected from fecal pet samples of companion animals attending the Veterinary Hospital UPVet – Porto, Portugal.

Isolate	Reason for veterinary visit	Antimicrobial resistance pattern	ESBL phenotype	Phylo group	β-Lactamase group or subgroup	Mutations in QRDR		PMQR
						gyrA	*parC*	
1	Otitis	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	Yes	A	OXA, CIT	Leu83Asn87	Ile80	–
2	Orthopedic surgery	AMP, CIP, TET, CTX, ATM, CEF, STR, NAL, SXT	No	B1	TEM, CTX-M-32	Leu83Asn87	Lys84	–
3	Pyoderma	AMP, FOX, TET, CTX, ATM, CAZ, AMC, CEF, NAL, SXT	Yes	A	TEM, CIT	Wild	Wild	*qnrS1*
4	Orthopedic surgery	AMP, CIP, TET, CEF, STR, NAL, CHL, SXT	Yes	A	TEM	Leu83Asn87	Ile80Val108	–
5	Check-up	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	Yes	A	OXA, CIT	Leu83Asn87	Ile80	–
6	Check-up	AMP, CIP, TET, ATM, CAZ, CEF, STR, NAL, CHL, SXT	Yes	B2	TEM, SHV, CIT	Leu83Tyr87	Ile80	–
7	Check-up	AMP, TET, ATM, CAZ, CEF, STR, CHL	Yes	D	CIT	Leu83	Wild	–
8	Check-up	AMP, CIP, TET, CTX, ATM, CAZ, CEF, NAL	Yes	D	CTX-M-1	Leu83Asn87	Ile80	–
9	Check-up	AMP, FOX, CIP, GEN, TET, CAZ, AMC, CEF, STR, NAL, CHL, TOB, SXT, KAN	Yes	A	OXA, CIT	Leu83Asn87	Ile80	–
10	Check-up	AMP, FOX, CIP, GEN, TET, CAZ, CEF, STR, NAL, CHL, TOB, SXT, KAN	Yes	A	OXA, CIT	Leu83Asn87	Ile80	–
11	Check-up	AMP, CIP, TET, CTX, ATM, CAZ, CEF, STR, NAL, SXT	Yes	D	TEM, CTX-M-1	Leu83Asn87	Ile80	–
12	UTI	AMP, CIP, GEN, TET, ATM, CAZ, CEF, STR, NAL, CHL, TOB, SXT, KAN	Yes	A	SHV, OXA	Leu83Asn87	Arg80	–
13	UTI	AMP, CIP, GEN, TET, ATM, CAZ, CEF, STR, NAL, CHL, TOB, SXT, KAN	Yes	A	SHV, OXA	Leu83Asn87	Arg80	–
14	UTI	AMP, CIP, GEN, TET, ATM, CEF, STR, NAL, CHL, TOB, SXT, KAN	Yes	A	OXA	Leu83Asn87	Arg80	–
15	UTI	AMP, CIP, GEN, TET, ATM, CEF, STR, NAL, CHL, TOB, SXT	Yes	A	SHV, OXA	Leu83Asn87	Arg80	–
16	Check-up	AMP, FOX, CIP, TET, ATM, CAZ, AMC, CEF, STR, NAL, CHL, SXT	Yes	B1	TEM, SHV	Leu83Asn87	Ile80	*qnrS1*
17	Ophtalmic disease	AMP, FOX, CIP, TET, CTX, ATM, CAZ, AMC, CEF, STR, NAL, CHL, SXT	Yes	A	TEM, SHV, CIT	Leu83Asn87	Ile80	–
18	Neurological disease	AMP, FOX, CIP, TET, CTX, ATM, CAZ, AMC, CEF, NAL	No	B1	TEM, CIT	Leu83Asn87	Ile80	–
19[a]	UTI	AMP, CIP, GEN, CTX, ATM, CEF, STR, NAL, CHL, SXT, KAN	Yes	D	TEM, CTX-M-55/79	Leu83Tyr87	Ile80	–
20	Plyuria and polydipsia	AMP, FOX, TET, CTX, ATM, CAZ, AMC, CEF, NAL, SXT	No	A	TEM, CIT	Wild	Wild	*qnrS1*
21	Skin Wound	AMP, CIP, TET, CEF, STR, NAL, CHL, SXT, KAN	No	A	TEM	Leu83Asn87	Thr56Ile80	–
22	Orthopedic disease	AMP, CIP, TET, ATM, CEF, NAL, CHL	Yes	A	SHV	Val83Gly87	Ile80	–
23	Orthopedic disease	AMP, TET, CEF, NAL	Yes	A	SHV	Val83	Ile80	–
24	Neurological disease	AMP, FOX, TET, AMC, CEF, STR, NAL, CHL, SXT	Yes	A	TEM, CIT	Leu83	Wild	–
25	Neurological disease	AMP, FOX, CIP, TET, ATM, CAZ, AMC, CEF, STR, NAL, CHL, SXT	Yes	A	TEM, SHV, CIT	Leu83Asn87	Ile80	–
26	Check-up	AMP, TET, CTX, CEF	Yes	D	TEM	Wild	Wild	–
27	Orthopedic disease	AMP, CIP, TET, CEF, STR, NAL, CHL	No	A	TEM	Leu83Asn87	Ile80	–
28	Diabetes mellitus	AMP, TET, ATM, CEF, CHL	Yes	D	SHV	Wild	Wild	–
29[a]	Hyperadreno corticism	AMP, CIP, GEN, CTX, ATM, CAZ, CEF, NAL, SXT, KAN	Yes	D	TEM, CTX-M-55/79	Leu83Tyr87	Ile80	–
30	Trauma	AMP, CIP, TET, STR, NAL, CHL, SXT	No	B2	TEM	Leu83Asn87	Ile80	–
31	Check-up	AMP, CIP, CAZ, CEF, NAL, CHL	Yes	D	SHV	Leu83Asn87	Ile80	–
32	Anemia	AMP, CIP, TET, CTX, CEF, STR, NAL, KAN	Yes	D	CTX-M-14	Leu83Tyr87	Ile80	–
33[a]	Orchiectomy	FOX, CTX, ATM, AMK, TOB	Yes	B2	CTX-M-2/44	Wild	Wild	–
34	Diarrhea	AMP, CIP, TET, CTX, CEF, NAL	Yes	A	CTX-M-15	Leu83Asn87	Ile80	–
35	Check-up	CIP, TET, NAL, SXT	No	B1	–	Leu83Asn87	Ile80	–
36	Check-up	AMP, CIP, STR, NAL, CHL, SXT	No	B1	–	Leu83Asn87	Ile80	–
37	Check-up	CIP, TET, STR, NAL, SXT, KAN	No	B1	–	Leu83Asn87	Ile80	–
38	Dysuria	AMP, CIP, TET, CTX, CEF, STR, NAL, SXT	Yes	B1	TEM, CTX-M-1	Leu83Asn87	Ile80	–

AMP, ampicillin; AMC, amoxicillin–clavulanic acid; ATM, aztreonam; CEF, cephalothin; CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; IPM, imipenem; GEN, gentamicin; AMK, amikacin; STR, streptomycin; TOB, tobramycin; KAN, kanamycin; CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; CHL, chloramphenicol; SXT, trimethoprim–sulfamethoxazol; NIT, nitrofurantoin.

[a] Direct sequencing of amplicons obtained did not allow differentiation between the two mentioned β-lactamase subgroups.

of several resistance determinants (Table 1). Of these 38 isolates, 34 and 36 displayed phenotypic resistance to quinolones and β-lactams, respectively, and 29 isolates had an ESBL phenotype (as shown by the method of disk approximation).

Regarding the phylogenetic group, all isolates yielded PCR products using the method described by Clermont and colleagues [21]. As a result, all of the isolates could be assigned to a phylogroup [26]. The majority of the isolates ($n = 19$) belonged to phylogenetic

group A, followed by group D ($n = 9$), group B1 ($n = 7$) and group B2 ($n = 3$).

3.2. Detection of β-lactamase genes

Two isolates out of the 38 did not present a β-lactam resistance phenotype and no *bla* genes were furthermore detected. Multiplex-PCR results revealed a β-lactam resistance genotype in 35 isolates out of the 36 isolates presenting phenotypic resistance to β-lactams; in one isolate none of the surveyed genes were detected, suggesting that other determinants may be present (Fig. 1). Three out of the 35 β-lactam-resistant isolates harbored β-lactamase genes belonging to three different groups; 17 β-lactam-resistant isolates had genes belonging to two groups and 15 β-lactam-resistant isolates presented a single gene. The groups of β-lactamases TEM, SHV, OXA, CTX-M-1/2/9 and a group of plasmid-mediated AmpC β-lactamase, CIT (including LAT, CMY and BIL genes), were detected in the isolates tested, as shown in Table 1. Groups CTX-M-8/25 and AmpC β-lactamase groups ACC, FOX, MOX, DHA, and EBC were not detected. The β-lactamase group TEM was the most frequently detected ($n = 18$) followed by CIT ($n = 12$), SHV ($n = 11$), OXA ($n = 8$), CTX-M-1 ($n = 7$) and CTX-M-2 and 9 ($n = 1$). Moreover, the nucleotide sequence analysis of the gene $bla_{\text{CTX-M}}$ gene revealed that the subgroups of CTX-M-1 were CTX-M-1 ($n = 3$), CTX-M-55/79 ($n = 2$), CTX-M-15 ($n = 1$) and CTX-M-32 ($n = 1$); the subgroups of CTX-M-2 and 9 were CTX-M-2/44 and CTX-M-14, respectively (Table 1). The nucleotide sequence of some amplicons did not allow for differentiation between two mentioned β-lactamase subgroups, CTX-M-55 or 79 and CTX-M-2 or 44.

3.3. Genetic determinants of quinolone resistance

The simultaneous presence of two mutations in the *gyrA* gene and one or two in the *parC* gene, reported to confer resistance to the action of CIP, was found in 30 out of the 34 isolates that presented phenotypic resistance to quinolones. Additionally, one of those isolates carried the plasmid-encoded quinolone resistance gene: *qnrs1* (Table 2). Out of those 30 isolates, 29 carried a transition TCG → TTG in position 83 of *gyrA*, which resulted in the substitution of serine by leucine (Ser83Leu) and one isolate carried a transversion and a transition TCG → GTG (isolate nr. 22, MIC = 8 μg/ml) leading to the substitution of serine by valine in position 83 (Ser83Val). Regarding the same gene, *gyrA*, we observed different substitutions of the aspartate in position 87. Twenty-five isolates presented the transition GAC → AAC (Asp87Asn), 4 isolates had the transversion GAC → TAC (Asp87Tyr), and one isolate showed the transition GAC → GGC (Asp87Gly). On the *parC* gene the mutations that we observed were a substitution of a serine by an isoleucine in position 80 (Ser80Ile) ($n = 25$) due to the transversion AGC → ATC ($n = 24$) or AGC → ATT ($n = 1$) and a substitution by arginine due to the transversion AGC → AGA ($n = 4$). In 2 isolates, a second mutation in *parC* was found, in position 108 ($n = 1$), where an alanine was substituted by a valine (GCG → GTG) (Ala108Val) (isolate nr. 4, MIC = 64 μg/ml) or in position 56 ($n = 1$) where alanine was substituted by threonine (GCC → ACC) (Ala56Thr) (isolate nr.21 MIC = 16 μg/ml). In position 84, 1 isolate harbored a point mutation with a transition of GAA → AAA that resulted in a substitution of glutamate by a lysine (Glu84Lys).

Four isolates presented resistance only to NAL and not to CIP; in 2 isolates the plasmid-encoded gene *qnrs1* was present and in the other 2 we detected the presence of at least one mutation in *gyrA* (Ser83Val or Ser83Leu) with ($n = 1$) or without ($n = 1$) the presence of one mutation in *parC* (Ser80Ile). Neither PMQR nor mutations in QRDR of *gyrA* and *parC* genes were detected in the 4 isolates out of the 34 that were phenotypically susceptible to quinolones.

4. Discussion

In the present study, 78 dogs who visited a veterinary hospital, either for a normal checkup or due to disease, were sampled in order to detect the presence of drug-resistant *E. coli* in their feces. A total of 38 multidrug-resistant isolates were selected for molecular characterization.

Regarding phylogenetic groups, half of the 38 multidrug-resistant isolates belonged to the phylogenetic group A, which comprises mainly nonpathogenic commensal strains [21]. Even so, the high number of AMRs those isolates carry is quite worrying. Accordingly, some studies of *E. coli* isolates from human urinary tract infections [27,28] and surgical wounds [29] have already reported a phylogenetic shift from group B2 to less virulent groups such as A (mainly), B1 and D; this change is accompanied by an increased prevalence of resistance to antimicrobials. Therefore, it seems that *E. coli* of phylogroup A are becoming associated with the presence of multidrug resistances. In addition, other studies have stated that integron-associated antibiotic resistance is less present in isolates from the phylogroup B2 [27].

Of the 38 multidrug-resistant isolates, 25 were collected from dogs that presented signs of disease. Consistent with other studies [8,11,30], isolates ($n = 10$) displaying an ESBL phenotype could also be collected from healthy animals. Although the inclusion criteria comprised the absence of antimicrobial administrations in the preceding four months, the lack of information about each animal's medical history, namely antibiotic treatments prior to that period, precludes the study of how these variables influenced the resistance selection.

Among the isolates with β-lactam resistance genotypes ($n = 36$), a high frequency of TEM, SHV, CTX-M and CIT determinants was observed. These β-lactamase groups are quite common and have been widely reported in humans and animals [8]. AmpC type β-lactamases were exclusively represented by the CIT group. This finding is in accordance with various studies that identified the subgroup CMY-2 in their sampled pets [8,31,32]. The sequence analysis was only performed on the CTX-M genes because of the interest in determining the subgroup of these ESBL genes, which are the most widespread among ESBLs and have been associated with different hosts and geographical distributions [33]. This fast screening method allowed for the identification of CTX-M-1 ($n = 3$), CTX-M-15 ($n = 1$), CTX-M-32 ($n = 1$) and CTX-M-14 ($n = 1$). Nevertheless, the amplicons obtained for groups CTX-M-1 and CTX-M-2 did not allow for distinction between CTX-M-55 and CTX-M-79 ($n = 2$) and CTX-M-2 and CTX-M-44 ($n = 1$), respectively.

We found that 41.7% of the isolates ($n = 15$) carried only one β-lactamase gene and 47.2% ($n = 17$) carried two genes. The genotypes TEM and both TEM and CTX-M-1 (13.9%) were the most frequently observed followed by SHV, OXA + CIT and TEM + CIT (11.1%). Some studies have characterized the resistance of extended-spectrum cephalosporins in clinical *E. coli* isolates from companion animals in the United States [34] and in Italy [31]. These investigations have also reported TEM in combination with CTX-M-1 to be the most predominant. CTX-M-15 was found in only one isolate, contrary to the abundance of this gene in the study of Huber et al. [35] and in a recent study of non-repetitive ESBL-producing *E. coli* isolates from dogs, cats and horses from other European countries, in which CTX-M-15 was the predominant ESBL type [10]. It is interesting to note that despite the widespread distribution of this gene among *E. coli* isolates from humans in Portugal [36], it is still very rare in isolates from companion animals [9], suggesting that either the expansion of *E. coli* clones among pets or the horizontal gene transmission between human and animal isolates may be slowly occurring. However, the $bla_{\text{CTX-M-32}}$ gene was found in 14 out of 45 *E. coli* ESBL producers among the gull population in the Porto area [7]. As a result, it seems that there is a higher occurrence of this

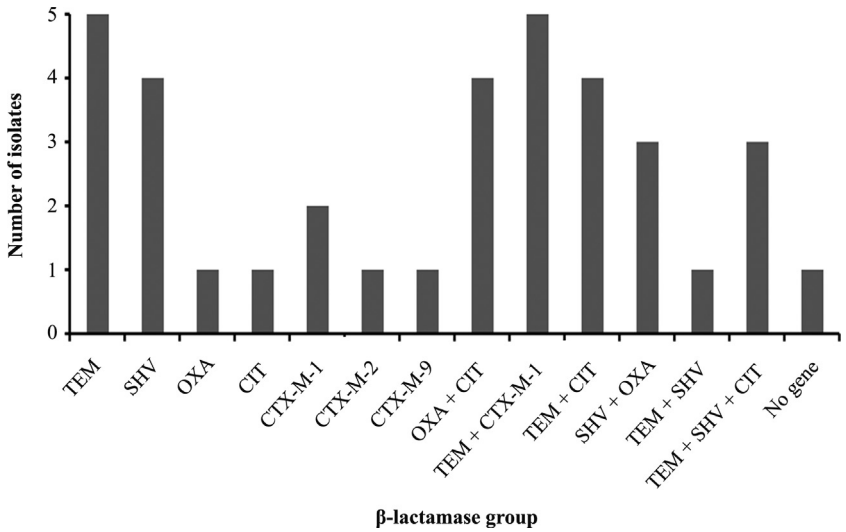

Fig. 1. Prevalence of the thirteen β-lactam resistance-conferring genotypes detected among 35 *E. coli* β-lactam-resistant isolates collected from fecal samples of dogs.

gene in gulls of this region than in pets who visited the veterinary hospital. Prior to 2012, CTX-M-14 was not detected in companion animals in Europe [34]. In this work we found one isolate harboring this gene.

Phenotypic resistance to quinolones was mainly due to mutations on the *gyrA* and *parC* genes, suggesting that this resistance phenotype is frequently vertically inherited. However, although to a lesser extent, horizontal gene transfer of resistance through *qnrS* was also detected. Resistance to CIP was determined by the presence of two mutations on the chromosomal gene *gyrA* and one or two mutations in *parC*. When resistance was restricted to NAL, we have verified the presence of the PMQR gene *qnrS* or the presence of one mutation in the chromosomal genes; such genotypes seem insufficient to confer resistance to CIP. However, resistance may not only be determined by the presence of *qnrS* gene; other factors (not evaluated) such as the possible presence of efflux pumps or organic solvent tolerance may also be responsible for the resistance [37].

The most frequent genotype ($n = 17$) conferring quinolone resistance was the result of a common double mutation on the QRDR of *gyrA*: substitution of a serine by a leucine in position 83 and aspartate by asparagine in position 87, accompanied by a single mutation on QRDR of *parC* with substitution of a serine by a isoleucine in position 80. We also detected a non-described mutation in *gyrA* due to a transversion and a transition (TCG → GTG) resulting in the substitution of a serine by a valine in position 83. However a valine in this position had already been reported [38]. This isolate had a MIC of 8 μg/ml to CIP. Moreover, in *parC*, we detected an unusual substitution of alanine by valine in position 108 [39], and in this isolate the MIC value was high (64 μg/ml). A substitution outside the QRDR was also identified (threonine in position 56) [40], in an isolate with a MIC of 16 μg/ml. Nonetheless, we cannot strictly state that this particular genotypes is solely responsible for conferring higher or lower MIC values; often there are other mechanisms that need to be considered. Whether these mutations have any significant effect on the MIC of CIP has not been investigated as part of our study. However, in previous studies a correlation between the number of mutations in *gyrA* and *parC* and the level of quinolone resistance was reported, where a single substitution in *gyrA* conferred low levels of resistance to CIP and a high level was associated with four mutations (two in *gyrA* plus two in *parC*) [39,41].

Curiously, ESBL genes were not frequently associated with PMQR. In fact, that association was only detected in three isolates, two harboring TEM and CIT and one harboring TEM and SHV. The PMQR gene, *qnrS*, encodes a protein capable of protecting type II topoisomerase from the action of quinolones and that results in low-level resistance; nevertheless it does not interfere with the selection of other resistance mechanisms [42]. Similarly, other studies [43] have found that fluoroquinolone resistance in uropathogenic *E. coli* isolates from companion animals was due to point mutations in QRDR that could be accompanied by PMQR mechanisms.

In conclusion, we have demonstrated the occurrence of a large number of multidrug-resistant *E. coli* in the feces of pets, as well as relevant resistance traits, pointing to companion dogs as being putative sources of dissemination of resistant bacteria and/or resistance determinants to other bacteria sharing the gastrointestinal tract, or to other animals/humans directly interacting with them.

Table 2
Prevalence of the eleven quinolone resistance-conferring genotypes detected among 34 quinolone-resistant *E. coli* isolates collected from fecal samples of dogs.

Mutations in *gyrA*	Mutations in *parC*	PMQR	Number of isolates	Quinolone resistance
Leu83Asn87	Ile80	–	17	CIP + NAL
Leu83Asn87	Lys84	–	1	CIP + NAL
Wild	Wild	*qnrS1*	2	NAL
Leu83Asn87	Ile80Val108	–	1	CIP + NAL
Leu83Tyr87	Ile80	–	4	CIP + NAL
Leu83Asn87	Arg80	–	4	CIP + NAL
Leu83Asn87	Thr56Ile80	–	1	CIP + NAL
Leu83Asn87	Ile80	*qnrS1*	1	CIP + NAL
Val83Gly87	Ile80	–	1	CIP + NAL
Val83	Ile80	–	1	NAL
Leu83	Wild	–	1	NAL

CIP, ciprofloxacin; NAL, nalidixic acid.

Hence, pets should not be disregarded in multisource studies related to human infections.

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