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Co-occurrence of clinically relevant β-lactamases and MCR-1 encoding genes in *Escherichia coli* from companion animals in Argentina

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ABSTRACT

Extended-spectrum β-lactamase (ESBL), plasmid-mediated AmpC (pAmpC) and MCR-1 phosphoethanolamine transferase enzymes have been pointed out as the main plasmid-mediated mechanisms of resistance to third generation cephalosporins (TGC) and colistin, respectively, and are currently considered a major concern both in human and veterinary medicine. Little data on these resistance determinants prevalence in companion animal infections is available. The aim of this study was to determine the resistance profile of *Escherichia coli* isolated from pet infections, in Argentina, and to characterize the resistance mechanisms to TGC, as well as the presence of the plasmid-borne colistin resistance gene, *mcr-1*. A total of 54 *E. coli* isolates were collected from clinical samples in dogs and cats; from them, 20/54 (37%, $CI_{95:}$ [24%; 51%]) displayed resistance to TGC. In this regard, thirteen pAmpC-producing isolates were positive for bla_{CMY-2} genes, whereas seven ESBL- producers harboured $bla_{CTX-M-2}$ ($n = 4$), $bla_{CTX-M-15}$ ($n = 2$) and $bla_{CTX-M-14}$ ($n = 1$) genes. One *E. coli* strain (V80), isolated from a canine urinary tract infection, showed resistance to colistin (MIC = 8 μg/ml) and whole-genome sequencing analysis revealed co-occurrence of *mcr-1.1*, $bla_{CTX-M-2}$, *aadA1*, *ant(2″)-Ia*, *catA1* and *sul1* genes; the former being carried by a 60,587-bp IncI2 plasmid, previously reported in human colistin-resistant *E. coli*. *E. coli* V80 belonged to ST770 and the highly virulent phylogenetic group B2. In general, most of these multidrug-resistant isolates belonged to the phylogenetic group F (11/20) and to a lesser extent B2 (5/20), B1 (2/20), D (1/20) and E (1/20). In summary, CMY- and CTX-M-type β-lactamases may constitute the main TGC resistance mechanism in *E. coli* isolated from pet infections in Argentina, whereas dissemination of colistin resistance mechanism MCR-1 in the human-animal interface has been mediated by IncI2 plasmids.

1. Introduction

A variety of β-lactams are currently licensed for use in veterinary medicine, and as any other antibiotics, provide the opportunity for selection of clinically relevant antimicrobial resistance. It is of concern the increase in reports relative on β-lactamases in bacteria recovered from animal origin (Li et al., 2007; Rubin and Pitout, 2014). Among those enzymes able to confer resistance to third generation cephalosporins (TGC), it is possible to find extended spectrum (ESBL) and plasmid-encoded AmpC-type (pAmpC) β-lactamases.

The CTX-M family are the most prevalent ESBLs worldwide (Cantón and Coque, 2006; Melo et al., 2018). These cefotaximases confer resistance to aminopenicillins, most TGC, cefepime and monobactams, and are efficiently inhibited by clavulanic acid. In human clinical isolates, resistance due to pAmpC β-lactamases is less frequent. In this case, a different pattern (cephamycin resistance but cefepime susceptibility, lack of inhibition by clavulanic acid) is present (Gutkind et al., 2013). Currently, CMY-type enzymes are the most common pAmpC among *Enterobacteriaceae*. In most cases, ESBL and pAmpC genes are located in plasmids which also carry genes encoding resistance to other antimicrobial classes, such as fluoroquinolones, aminoglycosides, sulfonamides and polymyxins (Dierikx et al., 2012; Gutkind et al., 2013).

The emergence of multidrug-resistant (MDR) pathogens and spread of antimicrobial resistance have increased in the last decade (Brown, 2015). Even if almost abandoned for decades, to date colistin (COL) is one of the last line therapy options against infections caused by MDR

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Gram-negative pathogens. The recent emergence of *mcr-1*, a plasmid-borne COL resistance gene, has alerted the public health systems and led to changes in how resistance is perceived globally. The *mcr-1* gene was first reported in 2015 in food, animals and human isolates from China (Liu et al., 2016); and since then, was explosively recognized in different bacterial species worldwide (Baron et al., 2016), even in Latin-American (Quiroga et al., 2018), where it seems to have been circulating unnoticed more than ten years ago.

Even if close relationship between pets and human beings clearly increases the potential risk on human health (Dierikx et al., 2012; Huber et al., 2013; O'Keefe et al., 2010; Wieler et al., 2011), the diversity and prevalence of resistance determinants in *Enterobacteriaceae* isolated from companion animals has been little explored as compared with plasmid-mediated antimicrobial resistance in *E. coli* isolated from humans and food-producing animals. In short, few studies were performed on *E. coli* isolates from dogs and cats in order to contribute to better understanding the global scenario of antimicrobial resistance (Melo et al., 2018; Shaheen et al., 2011).

The aim of this study was to determine the resistance profile of *E. coli* isolated from clinical samples of pets and to characterize the resistance mechanisms associated to TGC resistance by class A (ESBL) and class C (pAmpC) β-lactamases. Finally, the occurrence of the *mcr-1* gene among them was also investigated.

2. Materials and methods

2.1. Bacterial isolates

A total of 216 clinical specimens derived from infections of dogs and cats attended in different veterinary clinics in Buenos Aires, Argentina were collected during a 3-month period (April to June 2014). All specimens were sent in for microbiological diagnostic testing to a private veterinary laboratory established in the Buenos Aires city. Samples deemed contaminated by the laboratory or those polymicrobial were excluded.

Upon reception they were immediately spread onto MacConkey agar (Oxoid, Hampshire, England) plates and incubated at 37 °C for 24 h for screening of *Enterobacterales*. From them, 101 enterobacterial isolates from dogs ($n = 78$) and cats ($n = 23$) were identified by conventional biochemical methods and confirmed by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) with score values ≥ 2 (Bruker® Daltonics. Bremen, Germany) using the Bruker Biotyper software. All *E. coli* (n = 54), were stored in Tryptic Soy broth plus glycerol (20%) at −20 °C until further analysis.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was determined by disk diffusion method according to *Clinical Laboratory Standard Institute* (CLSI) recommendations.

The antibiotics tested were ampicillin 10 μg (AMP), amoxicillin-clavulanic acid 20/10 μg (AMC), cefotaxime 30 μg (CTX), ceftazidime 30 μg (CAZ), cefoxitin 30 μg (FOX), imipenem 10 μg (IPM), meropenem 10 μg (MEM), gentamicin 10 μg (GEN), amikacin 30 μg (AMK), trimethoprim-sulfamethoxazole 1.25/23.75 μg (TSM), nalidixic acid 30 μg (NAL), ciprofloxacin 5 μg (CIP) and levofloxacin 5 μg (LEV). Commercial antibiotic disks were acquired from Oxoid® and Britania S.A.® (Argentina). For GEN, breakpoints were those recommended for bacterial isolates from animals (CLSI VET01-S2, 2013) and for other antibiotics we used breakpoints as recommended for human isolates (CLSI M100-S27, 2017).

Minimum inhibitory concentration (MIC) of COL was performed on all *E. coli* isolates by broth microdilution according to European Committee on Antimicrobial Susceptibility Testing (EUCAST- v.7.1) (2019) and isolates with MIC > 2 mg/l were considered resistant.

Traditionally MDR is defined as non-susceptibility to at least 1 antimicrobial agent in 3 different antimicrobial families, but a different definition was proposed, in which different categories for the same antimicrobial family are considered independent. Just as an example using β-lactams, aminopenicillins plus β-lactamase inhibitors, non-extended spectrum cephalosporins (1 st and 2nd generation cephalosporins), cephamycins, extended-spectrum cephalosporins (3rd and 4th generation cephalosporins) and carbapenems belong into different categories. Here we adopted this MDR definition as non-susceptibility to ≥1 antimicrobial agent in at least 3 antimicrobial categories as defined herein (Magiorakos et al., 2012).

2.3. Phenotypic ESBL/AmpC testing

Screening for ESBL and pAmpC production were conducted by double disk synergy tests using CTX, AMC, CAZ and boronic acid 300 μg (AB) disks (Tsakris et al., 2009). The confirmatory test for ESBL production was initially tested on Mueller-Hinton agar by the standard diffusion method and placing both CTX and CAZ containing disks alone or with clavulanate (CA) (10 μg). The test was considered positive when an increase in the growth-inhibition zone around either CTX or CAZ disk containing CA was 5 mm or greater than the growth-inhibition zone diameter around the disk containing CTX or CAZ alone (Clinical and Laboratory Standards Institute. (CLSI, 2017). *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as control strains.

2.4. Detection and characterization of β-lactamase-encoding genes and the plasmid-encoded phosphoethanolamine transferase gene mcr-1

The prevalent ESBLs and pAmpCs in our country were investigated by PCR using specific primers (Table 1). In addition, the plasmid-encoded phosphoethanolamine transferase gene (*mcr-1*) was also detected by PCR. Briefly, three colonies from freshly prepared Tryptic Soy agar (TSA) plates were suspended in 200 μl of molecular biology grade water and boiled at 100 °C for 15 min. Cellular debris was removed by centrifugation at 15,000 x *g* for 5 min, and DNA-supernatant was used for PCR. The PCR mix (48 μl) was added to 2 μl of total DNA used as template. The PCR mix contained 0.2 mM deoxynucleoside triphosphate, 20 mM Tris–HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 μM of each primer and 1.25 U of T-free DNA polymerase (Inbio Highway, Argentina).

Amplicons were purified using ADN Puriprep-GP kit- Highway® and sequences were performed on an ABI 377 DNA sequencer (Perkin Elmer, Applied Biosystems). Sequences were aligned and corrected using the Vector NTI program. The nucleotides sequences were compared to previously described sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

2.5. Whole genome sequence analysis

For the MCR-1-producing *E. coli*, the total genomic DNA was extracted using a PureLinkTM Quick Gel extraction kit (Life Technologies, Carlsbad, CA) and genomic library was prepared using a Nextera XT DNA Library preparation Kit (Illumina Inc., Cambridge, UK) according to the manufacture´s instructions. WGS was performed using paired-end reads (150 bp) on a NextSeq platform (Illumina). Genome assembly was carried out using Velvet software v1.2.10 (Zerbino and Birney, 2008) and contigs were annotated using Prokka (www.github.com/tseemann/prokka). Multilocus sequence type (MLST), plasmid replicons, antimicrobial resistance and virulence genes were identified using the bioinformatics software/tools (MLST 2.0, PlasmidFinder 2.0, ResFinder 3.0 and VirulenceFinder 2.0, respectively), available from the Center for Genomic Epidemiology database (http://genomicepidemiology.org/).

The plasmid (pV80) was *in silico* closure using a hybrid strategy, which consists in aligning *de novo* contigs against NR database (NCBI) to find the best plasmid match and use this plasmid to generate a

Table 1
PCR primers used to detect β-lactamases and *mcr-1* genes.

Oligonucleotide name	Target gene	Sequence 5'→ 3'	Expected size (bp)	Reference
CTX-M-group-1F	$bla_{CTX-M-1}$	ATGGTTAAAAAATCACTGC	864	
CTC-M-group-1R		GGTGACGATTTTAGCCGC		
CTX-M-group-2F	$bla_{CTX-M-2}$	TTAATGATGACTCAGAGCATTC	902	Saba Villarroel et al., 2017
CTX-M-group-2R		GATACCTCGCTCCATTTATTGC		
CTX-M-group-9F	$bla_{CTX-M-9}$	ATGGTGACAAAGAGAGTGC	876	
CTX-M-group-9R		TCACAGCCCTTCGGCGATG		
CIT-M-F	bla_{pAmpC}	TGGCCAGAACTGACAGGCAAA	462	
CIT-M-R		TTTCTCCTGAACGTCGCTGGC		
MOX-MF	bla_{pAmpC}	GCTGCTCAAGGAGCACAGGAT	520	Pérez-Pérez and Hanson, 2002
MOX-MR		CACATTGACATAGGTGTGGTGC		
DHA-MF	bla_{pAmpC}	AACTTTCACAGCTGTGCTGGGT	405	
DHA-MR		CCGTACGCATACTGGCTTTGC		
CMY-F	bla_{pCMY}	ATGATGAAAAAATCGTTATGCT	1146	Cejas et al., 2012
CMY-R		TTATTGCAGCTTTTCAAGAATGCG		
CLR5-F	*mcr-1*	CGGTCAGTCCGTTTGTTC	309	Liu et al., 2016
CLR5-R		CTTGGTCGGTCTGTAGGG		

preliminary scaffold for after closing the remaining gaps using reads not used in the assembly process.

2.6. Phylogroup analysis and molecular typing of E. coli isolates

Main *E. coli* phylogenetic groups (A, B1, B2 or D) were assessed by PCR according to Clermont (Clermont et al., 2000) and compared with the improved version (Clermont et al., 2013) where new phylo-groups are detected. Clonal relatedness of these isolates was investigated by Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) and Repetitive Element Palindromic-Polymerase Chain Reaction (REP-PCR) (Versalovic et al., 1991). Isolates were considered to belong to the same clone if they could not be differentiated after examining their REP/ERIC-PCR patterns. Characterization of the O25b-ST131 clone of *Escherichia coli* was also done by allele-specific PCR assay based on detection of the *pabB* gene (Clermont et al., 2009).

2.7. Conjugation assay

Bacterial conjugation was performed by the solid mating-out assay using *E. coli* CAG 12177 strain (tetracycline-resistant) as a recipient and *E. coli* V80 as a donor. Transconjugants were selected on TSA plates supplemented with colistin (1 μg/ml) and tetracycline (30 μg/ml). The identity of putative transconjugants was confirmed using antimicrobial susceptibility testing, ERIC/REP-PCR and PCR detection of *mcr-1* and $bla_{CTX-M-2}$ genes. The conjugation transfer rate (R) was estimated through the formula "R = number of transconjugants / number of recipients".

3. Results

3.1. Identification of Enterobacterales isolates

From 216 total isolates recovered from sick pets, about one half (n = 101, 47%, CI_{95}: [40%; 54%]) were identified as *Enterobacterales*. Different species of *Enterobacterales* were confirmed by MALDI-TOF. Fifty four out of 101 (54%, CI_{95}: [43%; 63%]) isolates have been identified as *E. coli* (Fig. 1). Sources of these *E. coli* isolates were urine (n = 33), otitis (n = 7), anal sacs (n = 4), skin (n = 3), wounds (n = 3), vaginal secretion (n = 3) and preputial secretion (n = 1).

The remaining 47 isolates were identified as *Proteus mirabilis*, *Klebsiella pneumoniae, Enterobacter cloacae, Enterobacter* (now *Klebsiella) aerogenes* and *Serratia marcescens* (Fig. 1). They were recovered from urine (n = 23), otitis (n = 13), skin (n = 4), nasal cavity (n = 4), tracheal secretion (n = 1), vaginal secretion (n = 1) and preputial secretion (n = 1).

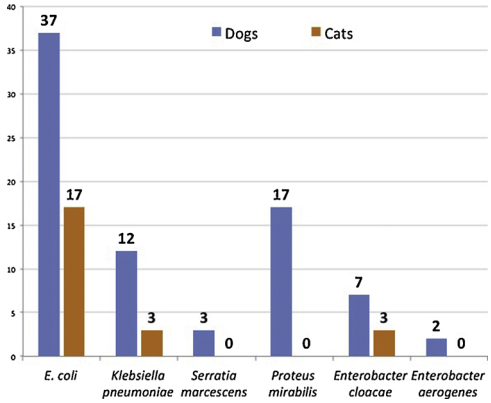


Fig. 1. *Enterobacterales* species recovered from sick dogs and cats in Buenos Aires, Argentina from April–June 2014 (n = 101).

3.2. Antibacterial susceptibility profile and characterization of β-lactamase in E. coli isolates

Twenty out of 54 *E. coli* isolates were resistant to TGC (CAZ and/or CTX (37%, CI_{95}: [24%; 51%]). These isolates were mainly recovered from urine 16/20 (80%, CI_{95}: [56%; 95%]) and to a lesser extent from content of the anal sacs 2/20 (10%, CI_{95}: [1%; 32%]), wound 1/20 (5%, CI_{95}: [0%; 25%]) and skin 1/20 (5%, CI_{95}: [0%; 25%]). Isolates recovered from urine samples 16/33 (48%, CI_{95}: [31%; 66%]) showed significantly higher resistance to TGC than isolates from other sources of infection 4/21 (19%, CI_{95}: [5%; 42%]) ($p = 0.027$) . No TGC-resistant strains were found in otitis, vaginal or preputial secretions.

Besides, one out of 54 *E. coli* (V80) (2%, CI_{95}: [0%; 10%]) was phenotypically categorized as COL- resistant (MIC = 8 μg/ml). This isolate corresponded to a dog urine sample.

By antimicrobial phenotypic test, most of TGC-resistant *E. coli* (13/20) were categorized as AmpC producers and the rest (7/20) as ESBL producers. All the thirteen AmpC-producing isolates were characterized as bla_{CMY-2} carriers, while 7 ESBL-producing isolates were characterized as $bla_{CTX-M-2}$ (4/7), $bla_{CTX-M-15}$ (2/7) and $bla_{CTX-M-14}$ (1/7) producers (Table 2). No carbapenem resistant isolates were observed.

Also, a high rate of quinolone resistance was observed in TGC-resistant *E. coli*, as 90% were resistant to NAL (CI_{95}: [68%; 99%]) and 75% to both CIP and LEV (CI_{95}: [50%; 92%]). Resistance to TMS was relatively high (60%, CI_{95}: [36%; 81%]). However, the percentage of isolates resistant to aminoglycosides was drug dependent (GEN, 55%, CI_{95}: [31%; 77%] and AMK, 10%, CI_{95}: [1%; 32%]).

In this study, 13/20 (65%, CI_{95}: [41%; 85%]) of TGC-resistant *E. coli*

Table 2
Characterization and antimicrobial susceptibility of ESBL/pAmpC producing *E. coli* in this study (n = 20).

Isolate	Animal	Disease	β-lactamase		Phylogroup[a]	Clonal relationship		Non suceptibility profile									MDR[b]	*mcr-1* gene
			ESBL	pAmpC		REP	ERIC											
V80[d]	Dog	Urine	CTX-M-2	-	B2/B2	III-a	V	AMP		CTX		GEN		NAL	CIP	LEV	COL	+/+	+
V91	Cat	Wound	CTX-M-2	-	B1/B1	III-b	II	AMP		CTX				NAL	CIP	LEV	TMS	+/+	-
V109	Cat	Urine	CTX-M-2	-	D/E	VII	VI	AMP	AMC	CTX				NAL	CIP	LEV		-/+	-
V111	Cat	Urine	CTX-M-2	-	B1/B1	V	II	AMP	AMC	CTX				NAL	CIP	LEV		-/+	-
V88[c]	Dog	Urine	CTX-M-14	-	D/B2	VI	VII	AMP		CTX				NAL	CIP	LEV		-/+	-
V39	Dog	Urine	CTX-M-15	-	D/F	I-a	I-a	AMP	AMC	CTX	CAZ	GEN	AMK	NAL	CIP	LEV	TMS	+/+	-
V1	Cat	Urine	CTX-M-15	-	D/F	I-a	I-a	AMP	AMC	CTX	CAZ	GEN		NAL	CIP	LEV	TMS	+/+	-
V3[c]	Dog	Anal sacs	-	CMY-2	B2/B2	VIII	IX	AMP	AMC	CTX	CAZ	FOX		NAL			TMS	+/+	-
V25	Dog	Urine	-	CMY-2	D/F	IX	VIII	AMP	AMC	CTX	CAZ	FOX	GEN	AMK	NAL	CIP	LEV	+/+	-
V27[c]	Dog	Skin	-	CMY-2	B2/B2	II	III	AMP	AMC	CTX	CAZ	FOX			NAL			-/+	-
V35[c]	Cat	Urine	-	CMY-2	B2/B2	IV	IV	AMP	AMC	CTX	CAZ	FOX					TMS	-/+	-
V51	Dog	Anal sacs	-	CMY-2	D/D	II	III	AMP	AMC	CTX	CAZ	FOX			NAL			-/+	-
V103	Cat	Urine	-	CMY-2	D/F	I-a	I-a	AMP	AMC	CTX	CAZ	FOX	GEN		NAL	CIP	LEV	TMS	+/+	-
V104	Cat	Urine	-	CMY-2	D/F	I-a	I-a	AMP	AMC	CTX	CAZ	FOX	GEN		NAL	CIP	LEV	TMS	+/+	-
V105	Cat	Urine	-	CMY-2	D/F	I-a	I-a	AMP	AMC	CTX	CAZ	FOX	GEN		NAL	CIP	LEV	TMS	+/+	-
V106	Cat	Urine	-	CMY-2	D/F	I-b	I-a	AMP	AMC	CTX	CAZ	FOX	GEN		NAL	CIP	LEV	TMS	+/+	-
V107	Cat	Urine	-	CMY-2	D/F	I-b	I-a	AMP	AMC	CTX	CAZ	FOX	GEN		NAL	CIP	LEV	TMS	+/+	-
V108	Cat	Urine	-	CMY-2	D/F	I-a	I-b	AMP	AMC	CTX	CAZ	FOX						-/+	-
V110	Cat	Urine	-	CMY-2	D/F	I-a	I-a	AMP	AMC	CTX	CAZ	FOX	GEN		NAL	CIP	LEV	TMS	+/+	-
V89	Cat	Urine	-	CMY-2	D/F	I-a	I-a	AMP	AMC	CTX	CAZ	FOX	GEN		NAL	CIP	LEV	TMS	+/+	-

AMP: ampicillin, AMC: amoxicillin-clavulanic acid, CTX: cefotaxime, CAZ: ceftazidime, FOX: cefoxitin, IPM: imipenem, MEM: meropenem, GEN: gentamicin, AMK: amikacin, TSM: trimethoprim-sulfamethoxazole, NAL: nalidixic acid, CIP: ciprofloxacin, LEV: levofloxacin and COL: colistin.
[a]triplex / quadruplex PCR "Clermont method" (Clermont et al., 2000, 2013).
[b]MDR: multidrug-resistant (Traditional / *Magiorakos et al.* definitions).
[c]O25b-ST131 and.
[d]ST 770.

were categorized as MDR according to the traditional definition. However, 100% of these strains were classified as MDR as defined by *Magiorakos* et al.

3.3. WGS analysis of MCR-1-producing E. coli V80 and plasmid characterization

The *mcr-1* gene was initially detected by PCR in COL-resistant *E. coli* (V80), which also harbors $bla_{CTX\text{-}M\text{-}2}$. WGS analysis of the *E. coli* V80 (DDBJ/ENA/GenBank accession number RZIF00000000) confirmed that this isolate belonged to ST770 (*adk*-52, *fumC*-116, *gyrB*-55, *icd*-101, *mdh*-113, *purA*-40, *recA*-38). Resistome analysis of V80 identified the β-lactam resistance gene $bla_{CTX\text{-}M\text{-}2}$, the aminoglycosides resistance genes *aadA1* and *ant(2″)-Ia*, the sulphonamide resistance gene *sul1*, the macrolide resistance gene *mph(B)*, and the phenicol resistance gene *catA1*. Furthermore, Col (MG828), Col156, ColpVC, ColRNAI, IncFIB, IncFII, and IncI2 plasmids were also identified.

The plasmid carrying the *mcr-1.1* gene (pV80, GenBank accession MH271383), which was estimated to be 60,587-bp in length, contained 83 open reading frames (ORF) with an average of 42.5% G + C content; it belongs into the IncI2 incompatibility group. Initially, pV80 was compared with the first description of *mcr-1* harboring plasmid (recovered from pig in China, GenBank accession KP347127) and displayed 99% coverage and identity (Fig S1 Supplementary material). When compared with other IncI2-plasmids that harbored the *mcr-1* gene distributed worldwide, it was observed that the overall backbone is conserved. Sequence alignment of pV80 with plasmids carrying *mcr-1* of human origin from Argentina (GenBank accession MF693349 and KY471314) has shown 86% coverage and 99% identity. Several deletions affecting the following genes: *dnaJ, nikB, pilV, virB5, tnpA and the relE/stbE* addiction system (Fig. 2a) were detected. The presence of a *tra* region, compatible with mobile plasmids, was confirmed by nucleotide comparison. As expected, pV80 was successfully transferred to *E. coli* CAG 12177 confirming that *mcr-1* is located on a conjugative plasmid. Transfer frequency of this plasmid was estimated in 1×10^{-5} per recipient cell. Other potentially transferable resistance genes were detected in this strain (*in silico*) but none of them co-located in the *mcr-1*-harboring plasmid.

Further analysis showed that the *mcr-1-pap2* element in pV80-IncI2 is integrated downstream of the *nikB* gene which encodes a relaxase. When compared with the human plasmid mentioned above, the *nikB* has a deletion of 48-bp (Fig. 2b) displaying 99% nucleotide identity (with 98% coverage) and 97% amino acids similitude. Although this deletion affects a repetitive amino acid region, NikB keeps both functional domains (called SMC y NBD94). On the other hand, *nikB* showed 100% of coverage and 99% of identity with the same region found in other plasmids described in isolates from other countries. Further inspection in the neighborhoods of the *mcr-1* gene failed to identify any direct or inverted repeat sequences suggesting the absence of any insertion sequence.

3.4. Phylogroup analysis and molecular typing

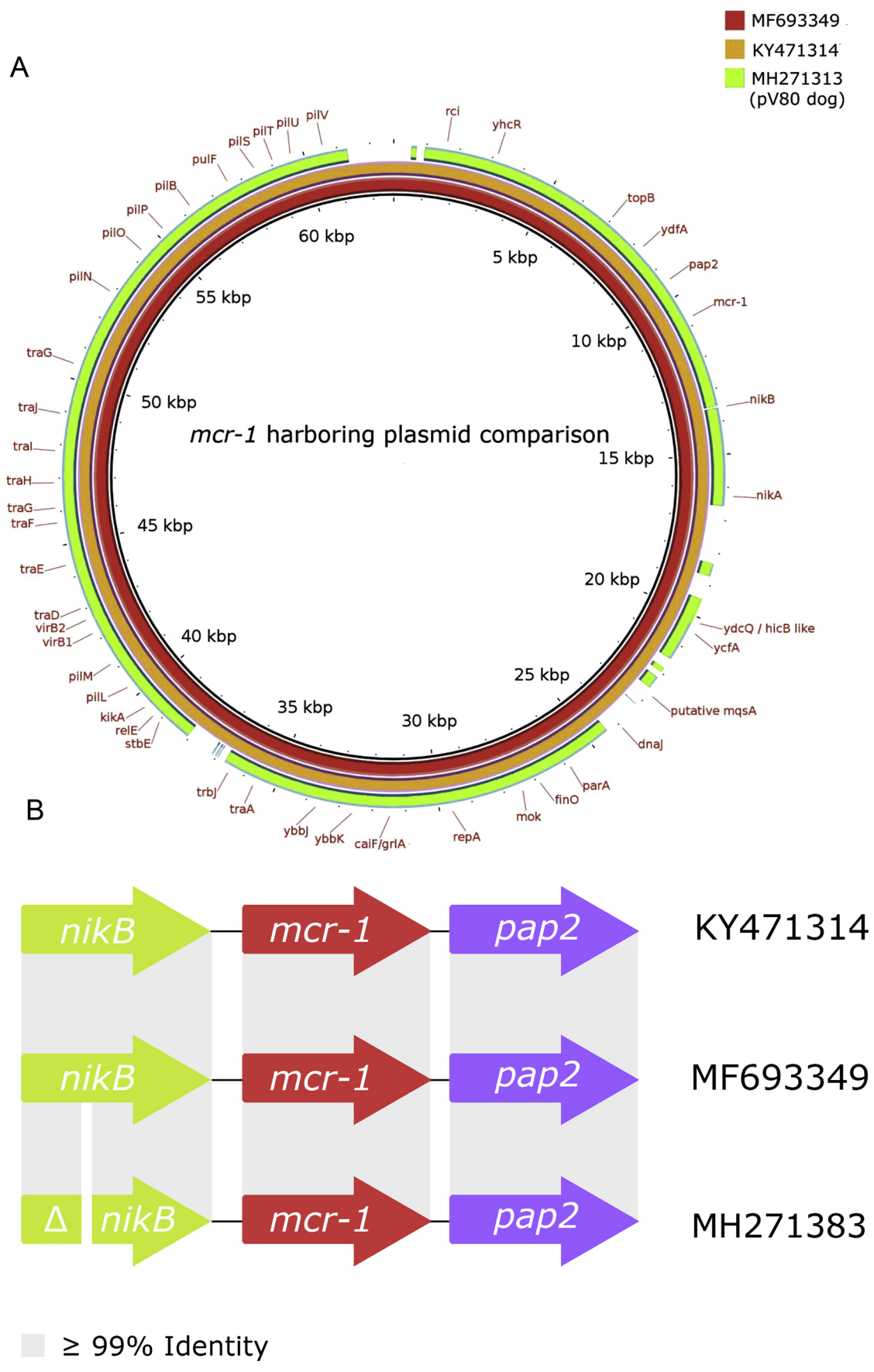
Overall, most TGC-resistant isolates belonged to the phylogenetic group F (11/20) and to a lesser extent B2 (5/20), B1 (2/20), D (1/20) and E (1/20) (Table 2). The isolates belonging to phylo-groups B1 and B2 maintained their classification with both methods of Clermont (triplex PCR and quadruplex PCR). In contrast, 11/14 phylo-group D *E. coli* identified by the triplex PCR could be reclassified as phylo-group F by quadruplex PCR.

Among 13 CMY-2 *E. coli* producers, 8 could be clustered within a single electropherotype by REP/ERIC-PCR belonging to the same phylogroup F and all of them were recovered from urine samples of cats; among them 7/8 strains showed the same accompanying resistance profile to GEN, NAL, CIP, LEV, TMS (Table 2).

Besides, four out of five *E. coli* belonging to phylogenetic group B2 were subsequently typed as O25b- ST131 (three of them CMY-2 producers, Table 2) and the remaining strain (V80) as ST770, as mentioned above.

4. Discussion

The increasing level of resistance in *Enterobacteriaceae* recovered from food-producing animals and pets has been recognized as an important public health problem during the last years (Ewers et al., 2012; Guerra et al., 2014). This study shows the characterization of resistance mechanisms to TGC in companion animals and to the best of our knowledge this is the first report in Argentina and South America. It is important to highlight that most TGC-resistant isolates were pAmpC producers (CMY-2) instead of ESBL producers, opposite to that



(caption on next page)

Fig. 2. Comparison the genetic structure of *mcr-1* harboring plasmids selected from *Escherichia coli* isolates from Argentina.
A) Comparative analysis of *Escherichia coli* strain 3216 plasmid pG3216 with two closely related *mcr-1*-harboring plasmids from *E. coli* isolates using the BLAST Ring Image Generator. The concentric rings display similarity between the reference sequence in the inner ring and the other sequences in the outer rings. MF693349 [*Escherichia coli* strain 3216 plasmid pG3216, *mcr-1* harboring plasmid from a rectal swab in a human neonate Intensive Care Unit in Buenos Aires, Argentina (Elena et al., 2018)]. KY471314 [*Escherichia coli* strain M19736 plasmid pMCR-M19736].
MH271383 [*Escherichia coli* strain V80 plasmid, *mcr-1* harboring plasmid from a urine sample from a dog in Buenos Aires, Argentina].
B) Comparison of the genetic context of *mcr-1*genes in different plasmids and sequences extracted from the GenBank database. Regions with ≥ 99 homology are indicated in gray shadow.

observed in human clinical isolates of *Enterobacteriaceae* in our country and worldwide where the ESBLs are the prevalent markers (Sennati et al., 2012; Melo et al., 2018).

In agreement with was described elsewhere (Smet et al., 2010) and even in Argentina (Cejas et al., 2012), CMY-2 was, by far, the main pAmpC β-lactamase identified in this work.

On the other hand, the most common ESBL detected in enterobacteria recovered from companion animals belong to the CTX-M family, in concordance with the prevalent ESBLs described in enterobacteria from human samples. CTX-M-producing enterobacteria are widespread among human populations but an increasing number of reports describe its presence in food animals and their environment (Ewers et al., 2012; Lazarus et al., 2015; Li et al., 2007). Although few CTX-M-harboring isolates could be recovered in this work, the three usual CTX-M groups circulating in our region (CTX-M-1, -2 and -9 groups) were detected in the expected rates (Sennati et al., 2012). In the literature, CTX-M type ESBLs and CMY enzymes were, until now, the main β-lactamases reported in *E. coli* isolated from companion animals and fortunately, carbapenemase-producing *E. coli* are still rare (Rubin and Pitout, 2014). Some studies carried out in Switzerland from clinical samples of pets identified the prevalence of CTX-M-15 among ESBL-producing *E. coli* (Huber et al., 2013; Zogg et al., 2018). It is worth noting that the detection of AmpC β-lactamases was not included in these studies. In contrast with these works, in a German resistance surveillance study led on companion and farm animals (Bft-GermVet monitoring program) found a lower rate (0.9%) of CTX-M-producing *E. coli* (CTX-M-1 and CTX-M-15) from respiratory and urinary tract infections recovered from dogs (Schink et al., 2011). In Brazil, the prevalence of ESBL/AmpC producing *E.coli* in pets with urinary tract infections was 8.3% (1/12) in cats and 8.1% (5/62) in dogs (Melo et al., 2018). A recent study conducted in Japan, detected 21% of TGC-resistant *E. coli* in pets with the CTX-M-1 and -9 groups as the main ESBLs identified (15%), and only to a lesser extent CMY-2 producers (5%) (Maeyama et al., 2018). Similar to our findings, O'Keefe et al. (2010) described in USA, that most isolates from urinary tract infection of canine and feline (35.3%) were CMY-producing *E. coli* (O'Keefe et al., 2010).

The growing number of infections caused by MDR pathogens isolated from clinical samples of companion animals is remarkable (Wieler et al., 2011). It is noteworthy that a high proportion of the TGC-resistant isolates, if not all, were considered MDR, highlighting the alarming emergence of ESBL/AmpC and MDR strains in pet populations within a defined geographic region in Buenos Aires, Argentina. Similarly, to our results, Zogg et al. (2018), have detected a high MDR prevalence (71.9%) among uropatogenic *E. coli* and more than half were moreover ESBL producers (Zogg et al., 2018).

In our study, most of TGC-resistant *E. coli* from dogs and cats belonged to phylogenetic group F. Phylotypes A and B1 are considered to be associated with commensal status or intestinal pathotypes, while B2, D and F are more commonly associated with strains causing extra-intestinal infections (Wagner et al., 2014).

Recently, non-related *mcr-1*-positive *E. coli* isolates from pets were reported in Beijing, however, plasmid characterization was not performed (Lei et al., 2017). During a multicentric surveillance study conducted in Brazil, COL resistance was detected in *Enterobacteriaceae* isolates from pets but *mcr-1* gene was not noticed (Fernandes et al., 2016). In this study, we report the first plasmid-borne colistin resistance gene (*mcr-1.1*) in *a* $bla_{\text{CTX-M-2}}$-producing *E. coli* recovered from companion animal in South America. The *mcr-1* gene was located in a transferable IncI2-plasmid such as it has been described previously in humans (Elena et al., 2018; Quiroga et al., 2018) and poultry farms (Dominguez et al., 2017, 2018) in our country.

It is remarkable the fact that this isolate was obtained in 2014, previous to the first description in the world of the *mcr-1* plasmid. The high similarity between the backbone sequences of *mcr-1* plasmids from different origins, suggest a possible common ancestor of the plasmid, but following by a different evolutionary path (according to deletions identified).

Antimicrobial resistance is a health issue with multifactorial origins and constitutes a global problem. Taking into account the close link that currently exists between human beings and pets, a good diagnosis and monitoring of antibiotic susceptibility is a critical aspect for the correct treatment of infections and epidemiological control. In these regards, it is not unlikely that TGC-resistant microorganisms can be exchangeable between animals and human.

Even if at the end of 2015, Argentina created a program of permanent surveillance of resistance in production animals such as cattle, pigs and chickens, the study of pets was neglected. However, in a true "one health" based strategy, a detailed knowledge of antimicrobial usage in humans, livestock and companion animal populations needs to be recorded. As such data are currently missing, improved simultaneous monitoring and surveillance programs are immediately needed (Ewers et al., 2012). Up to now, there were no data on the prevalence of ESBL and AmpC-producing *E. coli* and the circulation of the *mcr-1* plasmid in companion animals in our country.

5. Conclusions

The CMY-2 cephalosporinase constituted the main TGC resistance mechanism in our study, but cefotaximases belonging to three genetically different groups were also found, with the CTX-M-2 group as the most frequent. It is important to highlight the high level of resistance to different families of antibiotics (commonly used in pets) associated with TGC resistance. Ours findings demonstrate that *mcr-1* producing *E. coli* is also present in pets with clinical infections. As far as we know, this is the first description of an IncI2-plasmid carrying *mcr-1.1* in *E. coli* isolated from companion animals in South America. Our study also suggests that, in addition to food animals and humans, companion animals can also act as MDR *E. coli* reservoirs, adding another layer of complexity to the rapidly evolving epidemiology of plasmid mediated resistance in the community.

Conflict of interests

None of the authors has any conflict of interest related to this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.02.006.

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