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Brief Original Article

CTX-M-producing *Escherichia coli* Isolated from urban pigeons (*Columba livia domestica*) in Brazil

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Abstract

Introduction: Worldwide urban pigeons (*Columba livia domestica*) are an important reservoir of pathogenic and multidrug-resistant bacteria (MDR). Plasmids are key genetic elements in the dissemination of antimicrobial drug resistance in bacteria, including beta-lactams and quinolones, which are the most important classes of drugs for treatment of Enterobacteriaceae infections in human and veterinary medicine. The aim of this study was to determine the presence of *Escherichia coli* (*E. coli*) harboring plasmids containing extend-spectrum (ESBL) and pAmpC beta-lactamases, also plasmid-mediated quinolone resistance (PMQR) genes in urban pigeons from São Paulo State, Brazil.
Methodology: A collection of 107 isolates of *E. coli* from urban pigeons from four cities was screened by antimicrobial resistance phenotypic and PCR for genes encoding ESBL, pAmpC and PMQR genes. Clonality was evaluated by ERIC-PCR.
Results: We found three strains positive for $bla_{\text{CTX-M}}$ genes. In two clonally related CTX-M-8-producing strains, the gene was associated with IncI1 plasmids. An MDR strain harboring $bla_{\text{CTX-M-2}}$, the plasmid could not be transferred. No strain was positive for PMQR genes.
Conclusion: These results indicate that CTX-M-2 and CTX-M-8-producing *E. coli* are present in urban pigeons, which could serve as a reservoir for ESBL-producing *E. coli* in Brazil.

Key words: Synanthropic birds; antimicrobial resistance; ESBL; quinolone resistance; Enterobacteriaceae; *Escherichia coli*.

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Introduction

The emergence of resistant bacteria is a global public health concern [1]. In recent years, novel resistance mechanisms to various classes of antibiotics have begun to appear and are spreading worldwide. In this regard, extend-spectrum beta-lactamases (ESBLs) carry special importance, since they`re the first choice of treatment in human and animal infections. While many species of bacteria are reported as ESBL-producers, in *Escherichia coli* (*E. coli*), this class of beta-lactamases are broadly disseminated around the world [1,2].

Among the various classes of ESBL enzymes described, the most widespread in Enterobacteriaceae is the CTX-M family [1,3]. Many studies showed that *bla*CTX-M genes are present in large plasmids, often co-carrying other resistance genes, such as plasmid-mediated quinolone resistance (PMQR) genes [2,3]. In Brazil, as in most of the world, studies involving ESBL-producing strains are carried out on strains from humans, food-producing and pet animals, yet little is known about the carrying of this resistance determinant by synanthropic animals [4-6].

Domestic pigeons (*Columba livia domestica*) are feral urban birds adapted to live in diverse niches, occurring in all continents except Antarctica [7]. Pigeons are considered synanthropic birds due to their minimal feeding requirements and high prolific capacity, leading to overpopulation and conglomeration in urban areas, such as squares, parks, zoos, beaches, and places with food debris. Pigeons cohabit with humans, domestic and wildlife animals, and act as carriers of many emerging pathogens [8]. They have been associated with the occurrence of more than 60 species of bacteria, fungi, virus and protozoans [8,9].

Synanthropic birds also play an important role as a reservoir of multidrug resistant (MDR) strains. In an epidemiological survey evaluating the resistance profile in synanthropic birds in Spain, the authors reported that 33.3% of *E. coli* isolated from urban feral pigeons and 26% of *E. coli* from rural feral pigeons were MDR [10]. Some strains of *E. coli* presented class 1 integrons containing gene cassettes encoding for dihydrofolate

reductase A (*dfrA*) and aminoglycoside adenyltransferase A (*aadA*) [10]. Mobile genetic elements can be transmitted horizontally and are responsible for the spread of antimicrobial resistance genes and the emergence of resistant pathogens. The aim of this study, then, was to determine the presence of *E. coli* resistant to extend-spectrum cephalosporin and plasmid-mediated quinolone resistance (PMQR) in urban pigeons from São Paulo State, Brazil.

Methodology

Samples

Swabs of cloaca from 107 pigeons were collected in four cities of São Paulo State (São Paulo n = 20, Sorocaba n = 67, São Bernardo do Campo n = 12 and Santo Andre n = 8) between 2011 and 2016. The samples were selected by convenience-based criteria. This project was approved by the Ethics Committee of São Paulo University and authorized for scientific purposes (CEUA 5600130614). The material was transported to the Avian Medicine Laboratory to conduct microbiological isolation and culture. The samples were cultured overnight in brain – heart infusion broth (Difco – Laboratories, Detroit, MI, USA) at 37°C and isolated on MacConkey agar, after incubation at 37°C for 24 hours. One lactose-positive colony per sampled bird with morphology suggesting *E. coli* was selected, and identified by standard biochemical tests, using Enterokit® (Probac, São Paulo, Brazil).

Antimicrobial susceptibility tests

The 107 isolates were tested by disk-diffusion method, using commercial antibiotic disks (Cefar®, Cefar Diagnostic Ltda, São Paulo, SP, Brazil) to: cefotaxime (30 µg), amoxicillin-clavulanic acid (30 µg), ceftiofur (30 µg), cefoxitin (30 µg), sulfamethoxazole-trimethoprim (25 µg), nalidixic acid (30 µg), enrofloxacin (5 µg), ciprofloxacin (5 µg), streptomycin (10 µg), ampicillin (10 µg), amikacin (30 µg), gentamicin (10 µg), florfenicol (30 µg), and tetracycline (30 µg), as previous described [11,12]. *Escherichia coli* ATCC 25922 was used as a control. Production of ESBL was assessed by the double-disc synergism technique following the CLSI recommendations [8]. ESBL-producing strains and transconjugants were subjected to determination of minimum inhibitory concentrations (MIC) to cefotaxime, ceftazidime, cefepime, cefoxitin and ceftiofur, using agar dilution methods (mg/L) [11,12].

Detection of ESBL and PMQR genes, and determination of genetic environment

Strains presenting resistance to cephalosporins and/or quinolones (nalidixic acid, ciprofloxacin and/or enrofloxacin) were subjected to polymerase chain reaction (PCR) and sequencing to detect ESBL genes (bla_{TEM}, bla_{SHV} and bla_{CTX-M}), pAmpC genes (bla_{CMY}, bla_{ACT}, bla_{ACC}, bla_{FOX}, bla_{DHA}) [13], and PMQR genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *oqxAB*, *aac(6')lb-cr*, *qepA*) [14]. The DNA extraction was applied according to the protocol described by Boom *et al*. (1990) [15]. Reaction mixtures contained PCR buffer (1X), MgCl2 (1.5 Mm), 200 mM of each deoxyribonucleotides, 20 pMol of each primer, 1.0 U of Taq DNA polymerase, 5 µL of DNA template and ultrapure water until 50 µL. The amplification products were separated by electrophoresis on a 1.5% agarose gel and examined after staining with SYBER® Safe DNA Gel Satin (Invitrogen, São Paulo, Brazil). The molecular weight marker was 100 bp DNA ladder (LGC Biotechnology, São Paulo, Brazil).

Genetic environment of bla_{CTX-M} genes were determined by PCR mapping and sequencing using primers previous described [16]. Sequencing reactions was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit, followed by 36 cm capillary electrophoresis on polymer POP7 DNA analyzers. Analysis assembly and editing of nucleotide sequences were performed by the software Geneious v.9.1.8 (Biomatters Ltd, Auckland, New Zeland). The sequences were compared with known genes in the BLAST software (http://blast.ncbi.nlm.nih.gov/).

Phylogenetic group (Clermont method) and ERIC-PCR

Positive isolates were characterized by phylogenetic groups (Clermont method) [17]. The Clermont method classifies *E. coli* strains on phylogenetic groups (A, B1, B2, C, D, E, F, and Clade I), according to the amplification of genes *chu*A, *yja*A, *arp*A and TSPE4.C2 specific DNA fragment, by PCR.

The genotypic patterns of *E. coli* strains were evaluated by enterobacterial repetitive intergenic consensus (ERIC) after amplification of random distribution of inter-genome parts by PCR as described by Versalovic *et al*. (1991) [18].

Plasmid characterization

Transferability of genes was carried out by conjugation in *E. coli* $C600^{STR}$, with selection in agar containing streptomycin (2000 mg/L) and cefotaxime (2 mg/L). When conjugation assays failed, transformation was performed using *E. coli* TOP10 as

Table 1. Epidemiological, clonality, plasmids, genotypic and phenotypic characteristics of ESBL-producers strains and respective transconjugants.

Strains	Bird	City	Phylogroup	ERIC pattern	Plasmids (size)	β-lactamases	Resistance phenotype*					
							MIC (mg/L)					Disk diffusion
							CTX	CAZ	TIO	CPM	FOX	
ECpb02	02	Sorocaba	B1	I	IncI1 (~90kb)	CTX-M-8	>32	2	>32	>32	16	NAL
ECpb03	03	Sorocaba	B1	I	IncI1 (~90kb)	CTX-M-8	8	0,5	32	4	0,5	NAL
ECpb53	53	São Paulo	D	II		CTX-M-2	>32	16	>32	>32	8	NAL, ENR, CIP, GEN, STR, TET, SUL, SXT
Transconjugants/ receptor												
Tc-pb02					IncI1 (~90kb)	CTX-M-8	>32	8	>32	>32	4	-
Tc-pb03					IncI1 (~90kb)	CTX-M-8	>32	8	>32	>32	4	-
C600					-	-	0.125	0.25	0.5	0.125	2	-

*CTX=Cefotaxime, CAZ=Ceftazidime, TIO=Ceftiofur, CPM=Cefepime, FOX=Cefoxitin, NAL= Nalidixic Acid, ENR=Enrofloxacin, CIP=Ciprofloxacin, GEN=Gentamycin, STR=Streptomycin, TET= Tetracycline, SUL=Sulfonamides, SXT=Sulfa+Trimetoprim.

recipient strain. Plasmids were characterized by PCR-based replicon typing (PBRT) [19], and sizing determination was accomplished using the alkaline lysis extraction [20] following separation in a 0.8% agarose gel using plasmids of known sizes as references.

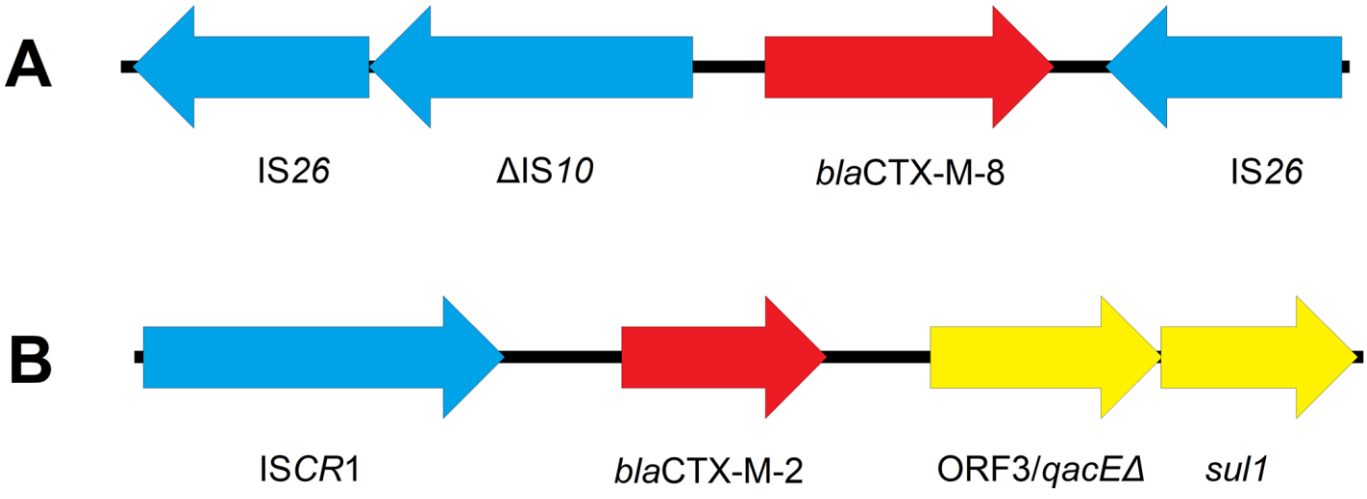
Results

Among 107 samples, all revealed positive growth for *E. coli*. Three strains were positive to $bla_{\text{CTX-M}}$ genes: two CTX-M-8 and one CTX-M-2, and none of the strains were positive for pAmpC and PMQR genes. The two CTX-M-8-producing isolates belonged to B1 phylogenetic group and were clonally related, presenting the same ERIC-pattern. In both of these strains, the conjugation of plasmids was successful. These plasmids belonged to IncI1 incompatibility group family and sized ~90Kb. In these transconjugants, only the resistance to beta-lactams was transferred, suggesting these plasmids harbor only the $bla_{\text{CTX-M-8}}$ gene (Table 1). After various conjugation and transformation attempts, the $bla_{\text{CTX-M-2}}$ gene of the third strain showed that it was not transferable. This strain belongs to phylogroup D (Table 1) and showed a multidrug-resistance phenotype, being resistant to quinolones, tetracycline, sulfas and trimethoprim, in addition to cephalosporins and ampicillin. As to genetic context, the $bla_{\text{CTX-M-8,}}$ gene was located in an IS*26* composite transposon, including a truncated copy of IS*10* transposase (Figure 1A). The genetic environment of $bla_{\text{CTX-M-2}}$, this gene was inserted in a class 1 integron with *sul1* and IS*CR*1 upstream, and orf3::*qacEΔ1* fusion gene downstream (Figure 1B).

Discussion

Pigeons are ubiquitous birds that have coexisted with humans for millennia [7]. Today, pigeons live in areas ranging from small villages to large metropolises, existing in close proximity to humans. These birds are known to act as reservoirs of pathogens, including

Figure 1. Genetic environment of $bla_{\text{CTX-M-8}}$ (A) and $bla_{\text{CTX-M-2}}$ (B). Red arrows representing $bla_{\text{CTX-M}}$ genes, blue arrows representing genes related to mobile genetic elements and yellow arrows representing other resistance genes.



virulent strains of *E. coli* [8]. However, little is known about the presence of antimicrobial resistant bacteria in these birds. In our study, we found urban pigeons carrying three ESBL-producing *E. coli* strains. These *E. coli* strains produce the enzymes CTX-M-2 and CTX-M-8, which are common among ESBL-producing Enterobacteriaceae from humans and food-producing animals in South America [3,21]

In the CTX-M-8-producing *E. coli* strains from pigeons, the gene was located on IncI1 plasmids. This plasmid family is a common vector of antimicrobial resistance genes and has a very efficient conjugative system [22]. IncI1 plasmids harboring $bla_{\text{CTX-M-8}}$ were previously described by Ferreira *et al*. (2014) [23] in *E. coli* from poultry in São Paulo State, Brazil. The authors showed that these strains belonged to phylogroups B1 and D, and $bla_{\text{CTX-M-8}}$ was located downstream of IS*10*. Here, we demonstrated that the gene was located between two copies of IS*26* and ΔIS*10* upstream, forming an IS*26* composite transposon (Figure 1). Dropa *et al*. (2016) [21] showed that IncI1 plasmids carrying $bla_{\text{CTX-M-8}}$ was present in *E. coli* isolated from public wastewater treatment plants in São Paulo, Brazil. These authors detected a similar genetic environment for $bla_{\text{CTX-M-8}}$, composing by IS*26* and IS*10*, although with different orientation of transposase genes. In a study conducted in China, IncI1 plasmids harboring a similar IS*26* composite transposon containing $bla_{\text{CTX-M-8}}$ was described in various *E. coli* isolates from poultry meat, including B1 phylogenetic strains [24]. Interesting, in that study, the poultry meat was imported from Brazil [24]. Another recent study of virulence and antimicrobial resistance in *E. coli* from captive pigeons in Brazil found a CTX-M-8-producing *E. coli* belongs to phylogenetic group B1, yet the genetic environment and plasmid was not investigated [25]. Comparing our results with the studies cited above, we suggest that phylogroup B1 *E. coli* strains harboring IncI1 plasmids could be considerate vectors of CTX-M-8 beta-lactamases in São Paulo State, independent of isolation source.

In the multidrug-resistant *E. coli* strain carrying $bla_{\text{CTX-M-2}}$, the gene was not mobilizable by conjugation or transformation. A study with *E. coli* from poultry of São Paulo State showed that in the ESBL gene of CTX-M-2-producing isolates, the gene could not be transferred because it was located on the chromosome of *E. coli* with different phylogenetic background [26]. In a previous study with *E. coli* strains from poultry that coproduce CTX-M-2- and CTX-M-55, we observed that $bla_{\text{CTX-M-55}}$ could be transferred, while $bla_{\text{CTX-M-2}}$ could not [27]. This phenomenon was also observed by Dropa *et al*. (2015) [28], who studied the transferability of CTX-M-2 in Enterobacteriaceae from humans, and obtained a low number of transconjugants/transformants. The PCR mapping of the genetic environment of $bla_{\text{CTX-M-2}}$ showed that the gene was inserted in a *sul1*-type class 1 integron, downstream a copy of IS*CR*1 (Figure 1B). This same genetic environment was described by Dropa *et al*. (2015) [28] in six Enterobacteriaceae species from Brazil, including *E. coli* strains isolated from human infections.

Conclusion

In summary, our study shows that the most common ESBLs from human clinical isolates, CTX-M-2 and CTX-M-8, are present in *E. coli* from urban pigeons, which could serve as a reservoir for ESBL-producing *E. coli* in São Paulo State, Brazil. These results reinforce the idea that a large population density of feral pigeons can harbor multidrug resistant bacteria, and disseminate these agents, especially in urban areas.

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