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BRIEF COMMUNICATION

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Colistin-resistant *Escherichia coli* belonging to different sequence types: genetic characterization of isolates responsible for colonization, community- and healthcare-acquired infections

Yrving Paiva[1], Debora Satie Nagano[1], Andre Luis Franco Cotia[1], Thais Guimarães[2], Roberta Cristina Ruedas Martins[1], Lauro Vieira Perdigão Neto[1], Marina Farrel Côrtes[1], Ana Paula Marchi[1], Louise Corscadden[3], Anna Silva Machado[4], Alexandre Inácio de Paula[2], Lucas Augusto Moyses Franco[1], Patricia Regina Neves[1], Anna Sara Levin[1], Silvia Figueiredo Costa[1]

ABSTRACT

The plasmid-mediated colistin-resistance gene named *mcr-1* has been recently described in different countries and it became a public health challenge. Of note, few studies have addressed the spread of *Escherichia coli* harboring the *mcr-1* gene in both, community and hospital settings. A total of seven colistin-resistant *E. coli* carrying *mcr-1*, collected from 2016 to 2018, from community (n=4), healthcare-acquired infections (n=2) and colonization (n=1) were identified in three high complexity hospitals in Sao Paulo, Brazil. These colistin-resistant isolates were screened for *mcr* genes by PCR and all strains were submitted to Whole Genome Sequencing and the conjugation experiment. The seven strains belonged to seven distinct sequence types (ST744, ST131, ST69, ST48, ST354, ST57, ST10), and they differ regarding the resistance profiles. Transference of *mcr-1* by conjugation to *E. coli* strain C600 was possible in five of the seven isolates. The *mcr-1* gene was found in plasmid types IncX4 or IncI2. Three of the isolates have ESBL-encoding genes ($bla_{CTX\text{-}M\text{-}2}$, n=2; $bla_{CTX\text{-}M\text{-}8}$, n=1). We hereby report genetically distinct *E. coli* isolates, belonging to seven STs, harboring the *mcr-1* gene, associated to community and healthcare-acquired infections, and colonization in patients from three hospitals in Sao Paulo. These findings point out for the potential spread of plasmid-mediated colistin-resistance mechanism in *E. coli* strains in Brazil.

KEYWORDS: *E. coli*. Colistin-resistance. *mcr-1*. Community and healthcare-acquired infections.

INTRODUCTION

In the last decade, colistin-resistance among Enterobacteriacae has increased all over the world and nowadays it is a public health challenge[1,2]. Until recently, colistin-resistance was related to chromosome mutations[1]. Moreover, a novel plasmid-mediated colistin-resistance gene, named *mcr-1,* was identified in *Escherichia coli* isolates from animals and humans in China, in 2015[2]. Since then, several reports have described *mcr* genes in animals bred for slaughter, from aquatic environments and in humans all over the world[3-8]. Soon afterwards, a study revealed travelers from South America colonized by *E. coli* harboring *mcr-1*[7]. Of note, few studies have addressed the spread of *E. coli* harboring the *mcr-1* gene in both, community and hospital settings.

[1]Universidade de São Paulo, Instituto de Medicina Tropical de São Paulo, São Paulo, São Paulo, Brazil

[2]Instituto de Assistência Médica ao Servidor Público Estadual de São Paulo, São Paulo, Brazil

[3]University of Leicester, Leicester, United Kingdom

[4]Hospital Totalcor, São Paulo, São Paulo, Brazil

Correspondence to: Silvia Figueiredo Costa
Universidade de São Paulo, Instituto de Medicina Tropical de São Paulo,
Av. Dr. Eneias Carvalho de Aguiar, 255,
CEP 05403000, São Paulo, SP, Brazil

E-mail: silviacosta@usp.br

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Our study described the clinical and molecular characteristics of community and healthcare-acquired infections as well as isolates from colonization caused by colistin-resistant *E. coli* carrying *mcr-1* in three Brazilian hospitals.

MATERIALS AND METHODS

Colistin-resistant *E. coli*, from seven clinical samples, urine (n=5), feces (n=1), and surgical wound secretion (n=1), were identified in three high complexity hospitals, in Sao Paulo, Southeastern Brazil, between March, 2016 and January, 2018. Hospital A is a highly complex General Hospital with 800 beds, Hospital B is a tertiary General Hospital with 721 beds and Hospital C, with 170 beds, is specialized in cardiology and is predominantly surgical.

Data regarding demographic and clinical characteristics of all patients, including travels in the last year, contact with animals and rural area exposure, were retrieved from medical records, and an EPIINFO® database (CDC-USA 2017) was built. Clinical and laboratory data are stored at the Medical Research Laboratory LIM49, at Hospital das Clinicas. Feces positive for *E. coli* harboring *mcr-1* were considered positive. This study was approved by the Ethics Committee of Hospital das Clinicas, University of Sao Paulo, Brazil (approval Nº 2.452.282).

The antimicrobial susceptibility test was performed using the Sensititre (Thermo Fisher Scientific, Cleveland, USA). The Minimum Inhibitory Concentration (MIC) for colistin was determined by the broth microdilution method as well[8]. MIC values > 2 mg/L were considered indicative of colistin-resistance, according to the Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST)[9].

The pattern of resistance to polymyxins has been considered as suggestive that the isolate harbors the *mcr-1* gene. The *mcr-1* gene was screened using the Polymerase Chain Reaction (PCR) as described previously[2] and confirmed by Sanger's sequencing within the NCBI database. The seven isolates were submitted to Whole Genome Sequencing (WGS) using the platforms MiSeq Illumina™ (Illumina Inc., San Diego, CA, United States)[10] or Ion Torrent (Thermo Fisher Scientific, Waltham, MA, USA) technologies[11]. *De novo* assemblies were carried out using SPAdes v.3.11.1 (Center for Algorithmic Biotechnology, St. Petersburg, Russia)[12]. The sequence type (ST) was checked by MLSTFinder tool (Multilocus Sequence Typing) and confirmed using the database PubMLST[13]. Plasmids, resistance and virulence genes were analyzed using PlasmidFinder v. 2.1[14], ResFinder v. 4.1[15] and VirulenceFinder v. 2.0[16] tools. The type of plasmids harboring the *mcr-1* gene was determined by analysis on Geneious v. R11 (University of California, CA, USA)[17]. The phylogenetic tree was constructed with the seven sequenced genomes based on SNPs using the REALPHY version 1.12 with default parameters[18]. Briefly, sequences were mapped to the reference sequence (*E. coli* ATCC25922) via Bowtie2. From this alignment, SNPs were calculated using Seaview[19] and multiple sequence alignments were recreated using PhyML with 500 bootstrap replications for the tree construction[18]. The genome of the reference isolate was used as an outgroup to root the tree.

The conjugation experiment was performed with an streptomycin-resistant lactose-negative colistin-susceptible *E. coli* C600 strain as the recipient, in Luria Bertani broth medium. Transconjugant selection was executed on MacConkey agar plate with streptomycin (2 mg/mL) and colistin (2 µg/mL). Gene mobilization was confirmed by PCR.

RESULTS

The mean age of the patients was 64 years old (ranged from 3 to 91 years old) and five of the seven were female. Most patients presented with urinary tract infections (UTI) (5/7) (Table 1). Four of five UTI strains were identified within 24 h of admission. Only one patient had a history of exposure to a rural area, with breeding of animals and consumption of meat. Two others had no history of exposure to the countryside or travel in the last year (Table 1). Although five strains were from hospital B, the patients were not from the same family, neither had epidemiological links. Patients were on contact precaution until hospital discharge.

Each of the seven *E. coli* isolates belonged to a different ST (ST744, ST131, ST69, ST48, ST354, ST57, ST10) (Figure 1). In total, 163,155 SNPs were analyzed in the phylogenetic tree and the number of SNPs is identified in each node (Figure 1). No cluster pattern was observed with the exception of isolates from hospital A and C (ST44 and ST10) that grouped closer with 5,104 SNPs of difference (Figure 1).

The isolates differed regarding the resistome varying up to nine antimicrobials to which isolates were resistant (Table 1). The most relevant mechanisms of resistance detected were genes that encode betalactamases (bla_{TEM-1B}; bla_{OXA-1}; bla_{CMY-2}; $bla_{CTX-M-2}$; $bla_{CTX-M-8}$), aminoglycosides modifying enzymes (*aadA1; aadA2; aadB; aac(3)-IId; aac(3)-VIa; aph(3')-Ia; aac(6')Ib-cr; aadA5; aph(6)-Id; aph(3'')-Ib*), and genes that can lead to resistance to macrolides (*mph(A)*), chloramphenicol (*catA1; catB4; floR*), sulfonamides (*sul1; sul2; sul3*); tetracyclines (*tet(A); tet(B)*) and quinolones (*qnrS1*).

Table 1 - Characteristics of seven infected or colonized patients and of each *E. coli* strain harboring *mcr-1* from three Brazilian hospitals, between 2016-2018.

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Gender	Female	Female	Male	Female	Female	Female	Male
Age (in years)	55	87	57	82	77	91	3
Hospital	A	B	C	B	B	B	B
Distance from Hospital A	NA	6.2 km	2.7 km	6.2 km	6.2 km	6.2 km	6.2 km
Unit	Emergency Room	Emergency Room	Nursery	Emergency Room	Emergency Room	Emergency Room	Pediatric intensive care
Date of Isolation	March 2016	November 2016	August 2016	April 2017	May 2017	December 2017	January 2018
Source	Feces	Urine	Postoperative Wound Secretion	Urine	Urine	Urine	Urine
Underlying Diseases	Hepatic encephalopathy, diabetes	Urinary tract infection	Myocardial revascularization (postoperative wound infection), diabetes	Atrioventricular block, urinary tract infection	Urinary tract infection	Alzheimer, chronic renal failure, breast cancer	Neurologic disease
Rural Area Exposure	Yes	Unknown	No	Unknown	Unknown	Unknown	No
***E. coli* ST**	ST744	ST131	ST10	ST69	ST48	ST354	ST57
GenBank accession number	JAATKQ000000000	VHOI00000000	JAATKV000000000	JAATKU000000000	JAATKT000000000	JAATKS000000000	JAATKR000000000
COL MIC	**>4 µg/mL**	**4 µg/mL**	**>4 µg/mL**	**>4 µg/mL**	**>4 µg/mL**	**>4 µg/mL**	**>4 mg/mL**
MER MIC	<1 µg/mL	<1µg/mL	<1 µg/mL	<1 µg/mL	<1 µg/mL	<1 µg/mL	<1 µg/mL
MIC of other antimicrobials (µg/mL) (Resistance)	DOX (>16), GEN (>8), TOB (>8), SXT (>4/76), LEVO (>8), TAZ (16), FOT (8), A/S (32/16), TIM (128/2)	DOX (16), GEN (>8), TOB (>8), LEVO (>8)	DOX (16), SXT (>4/76), AZT (>16), FEP (>16), FOT (>32), A/S2 (>64/32), P/T (>64/4), TIM (>128/2)	SXT (>4/76), A/S (32/16)	TOB (>8), FEP (16), FOT (32), A/S (64/32)	DOX (16), SXT (>4/76), LEVO (>8), FOT (32), A/S (32/16)	GEN (>8), SXT (>4/76), LEVO (>8), AZT (16), FOT (>32), A/S (32/16)
Resistance Genes	*aadA1; aac(3)-VIa; aph(3')-Ia; aac(6') Ib-cr; aadA5; aph(6)-Id; aph(3")-Ib; blaTEM-1B; blaOXA-1; blaCMY-2.* ***mcr-1****; aac(6')-Ib-cr. MLS: mph(A); catB4; catA1; sul2; sul1; tet(B); dfrA14; dfrA17*	*aac(3)-IId; aph(6)-Id; blaTEM-1B; mph(A); sul2; tet(A);* ***mcr-1******	*aph(3')-Ia; aadA2; blaCTX-M-8; blaTEM-1B;* ***mcr-1****; lnu(F); floR; qnrS1; sul3; tetA; drfA12*	*aadA5; blaTEM-1B;* ***mcr-1****; mph(A); sul1; sul2; dfrA17*	*aadB; aadA1; blaCTX-M-2;* ***mcr-1****; sul1*	*aadA1; aadB; strB; strA; blaTEM-1B;* ***mcr-1****; mph(B); sul1; sul2; tet(A); dfrA1*	*aadB; aadA1; blaCTX-M-2;* ***mcr-1****; sul1; sul2; dfrA1*
Plasmids detected (replicon linked to *mcr*-1)	IncI1; IncQ1; **IncX4**; IncFIB(AP001918); IncFIC(FII)	IncFII(pRSB107); IncFIA; Col(BS512); IncFII(pHN7A8); IncFIB(AP001918); IncX1; (**not determined**)	**IncX4**; IncI1; IncFIA; IncFIB(AP001918); IncX1; IncFII(pRSB107); IncR	IncFIB(AP001918); IncFII(29); **IncX4**; Col156	ColRNAI; Col(MG828); IncA/C2; IncFIC(FII); IncI1; **IncI2**; IncFIB(AP001918).	p0111; IncQ1; **IncX4**; IncFII; ColRNAI	**IncI2**; IncFIB(AP001918); IncFIC(FII); Col(MG828)
***mcr-1* detected in transconjugant**	Yes	No	Yes	Yes	No	Yes	Yes
Virulence Factors	*iroN; tsh; cma; iss; gad*	*gad; sat; iha; iss*	*iss*	*sat; iha; gad; eilA; iss; lpfA; senB*	*iss; iroN; mchF; tsh; gad*	*mcmA ; mchF; lpfA; eilA; air; cma; gad*	*Iss; iroN; tsh; cma; gad*

NA = not applicable; COL = colistin; MER = meropenem; DOX = doxycycline; GEN = gentamicin; TOB = tobramycin; SXT = trimethoprim-sulfamethoxazole; LEVO = levofloxacin; TAZ = ceftazidime; FOT = cefotaxime; A/S = ampicilin-sulbactam; TIM = ticarcilin-clavulanate; FEP = cefepime; AZT = aztreonam; P/T = piperacilin-tazobactam. *iroN* (enterobactin siderophore receptor protein); *tsh* (temperature-sensitive hemagglutinin); *cma* (colicin M); *iss* (increased serum survival); *gad* (glutamate decarboxylase); *sat* (secreted autotransporter toxin); *iha* (adherence protein); *eilA* (salmonella HilA homolog); *lpfA* (long polar fimbriae); *senB* (plasmid-encoded enterotoxin); *mchF* (ABC transporter proein MchF); *mcmA* (microcin M part of colicin H); *air* (enteroaggregative immunoglobulin repeat protein). *-*mcr-1* gene in patient 2 was detected only by PCR and Sanger's sequencing (not by Whole Genome Sequencing).

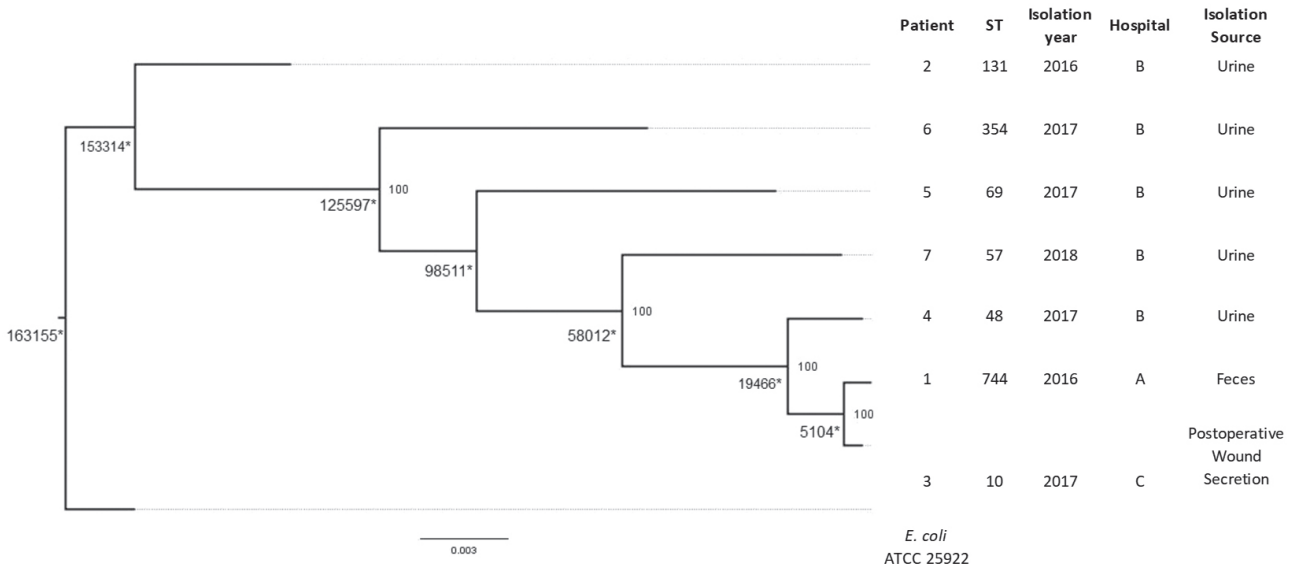

Figure 1 - Maximum likelihood phylogenetic tree of the *E. coli* genomes. The tree was rooted using the reference genome ATCC25922. SNPs are identified in each node with an asterisk (*).

In isolates from patients 1, 3 and 6, *mcr-1* and IncX4 replication initiation genes were identified in the same contigs, and from patient 4 and patient 7 with IncI2 replication gene. The isolate from patient 5 presented with *mcr-1* and replication initiation genes in different contigs. Nevertheless, the *mcr*-1 gene was detected on the transconjugant of the isolate from patient 5, a type of carrier plasmid that could not be determined by WGS. Transference of *mcr-1* by conjugation was possible in five of the seven isolates, the lactose-negative *E. coli* C600 transconjugants were streptomycin-resistant (as expected), but also colistin-resistant (Table 1). The *mcr-1* gene in the isolate from patient 2 was detected only by PCR and confirmed by Sanger's sequencing, but not by WGS, which could be due to the loss of gene or an impairment of the sequencing platform, as it was the only isolate sequenced by the Ion Torrent technology.

DISCUSSION

We tested phenotypically and genotypically seven isolates of colistin-resistant *E. coli* from seven different colonized or infected patients from three Brazilian hospitals. The isolates have other antimicrobial resistance genes, among which genes encoding extended-spectrum beta-lactamases (ESBL) ($bla_{\text{CTX-M}}$ variants) and AmpC cephalosporinase (bla_{CMY}) are frequently transmitted by conjugation.

Among the seven different STs, six have already been assigned to isolates harboring *mcr-1* in other countries[6,20-22]. We detected *mcr-1* in IncX4 plasmids of the isolates from four patients (Table 1), similar to some previously reported in Brazil[23,24], and in Incl2 plasmids of the isolates from two patients (Table 1), similar to the first isolate described in China[2]. Transference by conjugation was not possible with isolates from patients 2 and 4, which could be related to carriage by plasmids not as easily transferable as the IncX4 type or impairment in transfer mechanisms. In fact, a previous study reported this challenge, as the *mcr-1* gene was successfully transferred to *E. coli* strain C600 only in 34% of the studied isolates[25].

Resistance to colistin usually rises from selective pressure due to the use of polymyxins[2]. In Brazil, colistin was widely used as a growth promoter in animal production until 2016, when it was banned[26] due to identification of plasmid-mediated colistin-resistant isolates in the country. It is important to point out that the prevalence of colistin-resistance isolates can be underestimated, as colistin-susceptibility is not routinely tested in outpatients' clinical samples.

Up to now, the *mcr-1* gene is the most frequent plasmid-mediated colistin-resistance gene reported in Brazil[26,27]. It has been described in *Salmonella enterica* isolated from poultry meat[21], from food[27], and in *E. coli* from cattle and humans[28,29] and *Klebsiella pneumoniae* from humans[29].

At first, susceptibility to antimicrobials besides polymyxins was frequently observed in isolates harboring the *mcr-1* gene. However, the reports of isolates with resistance mechanisms to other antimicrobials, such as cephalosporins and carbapenems, are increasing[24,28]. In our study, all the strains were susceptible to carbapenem, but carried genes encoding other beta-lactamases (bla_{OXA}, bla_{TEM}, $bla_{\text{CTX-M}}$ and bla_{CMY}), frequently transmitted by conjugation[28] as well as aminoglycosides modifying

enzymes (*aadA1; aadA2; aadB; aac(3)-IId; aac(3)-VIa; aph(3')-Ia; aac(6')Ib-cr; aadA5; aph(6)-Id; aph(3")-Ib*), and genes that can lead to resistance to macrolides (*mph(A)*), chloramphenicol (*catA1; catB4; floR*), sulfonamides (*sul1; sul2; sul3*); tetracyclines (*tet(A); tet(B)*) and quinolones (*qnrS1*).

The SNPs tree and the seven STs described showed the diversity of *E. coli* harboring *mcr-1* in Brazil. Horizontal *mcr-1* gene transfer is supported by the SNP analysis as the strains were not clonal, as expected from different STs. Moreover, they were not closely related in regard of the hospital which they were isolated from or by year, supporting their acquisition from different sources and/or periods. Also, these results highlight the potential of colistin-resistance horizontal dissemination, and the possible spread of different STs capable of harboring plasmids with *mcr-1* in the community, since five isolates were collected in emergence departments, and four were identified within 24 hours of admission. However, acquisition in previous hospitalizations cannot be discarded. Therefore, our findings alert for the potential dissemination of *mcr-1* in the country and emphasize the need to rethink strategies to control the colistin-resistance, such as surveillance for *E. coli* strains, screening for *mcr-1* at emergency departments and in the food chain.

An important limitation of our study was that, although, the *mcr*-1 of isolate 2 was detected by PCR and confirmed by Sanger's sequencing, it was not found using the Ion-Torrent platform. It will be important to confirm our finding using a different new generation sequencing platform such as Illumina.

In summary, we reported genetically distinct *E. coli* isolates harboring the *mcr-1* gene associated to community- and healthcare-acquired infections and colonization in patients from three hospitals in Sao Paulo. The strains belonged to different STs. Our findings pointed out for the potential dissemination of this plasmid-mediated colistin-resistance mechanism in *E. coli* strains.

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