

# CTX-M-15-producing uropathogenic *Escherichia coli* isolates at Rio de Janeiro, Brazil: Molecular epidemiology and MALDI-TOF MS

Thiago Pavoni Gomes Chagas<sup>1</sup>, Luís Felipe da Silva Caldeira<sup>2</sup>, Alexandra Vidal Pedinotti Zuma<sup>3</sup>, Ivson Cassiano de Oliveira Santos<sup>4</sup>, Ana Paula D'Alincourt Carvalho-Assef<sup>4</sup>, Marise Dutra Asensi<sup>4</sup>, Flávia Lúcia Piffano Costa Pellegrino<sup>2</sup>

<sup>1</sup>Departamento de Patologia, Faculdade de Medicina, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil;

<sup>2</sup>Laboratórios Integrados de Pesquisa em Bactérias Resistentes aos Antimicrobianos, Unidade de Farmácia, Centro Universitário Estadual da Zona Oeste-UEZO, Campo Grande, Rio de Janeiro, Brazil;

<sup>3</sup>Seção de Análises Clínicas, Hospital de Aeronáutica dos Afonsos, Rio de Janeiro, Brazil;

<sup>4</sup>Laboratório de Pesquisa em Infecção Hospitalar, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, Brazil

## SUMMARY

Because of the high prevalence of CTX-M-15-producing *Escherichia coli* isolates causing urinary tract infections in Rio de Janeiro, we have investigated *bla*<sub>CTX-M-15</sub> gene presence, as well as CTX-M-15 production, in 32 *E. coli* isolates recovered from the urine of outpatients assisted at a public hospital located in the west zone of Rio. Molecular epidemiology was assessed by PFGE and phylo-typing methods. The work highlights the good performance of MALDI-TOF MS as an alternative tool to detect extended-spectrum beta-lactamases among CTX-M-15-producing *E. coli* isolates.

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CTX-Ms constitute the largest group among extended-spectrum beta-lactamases (ESBLs), globally disseminated (Bevan *et al.*, 2017). Although in South America, the CTX-M-2 variant is still significant (Villegas *et al.*, 2008), in Brazil, the *Escherichia coli* carrying *bla*<sub>CTX-M-15</sub> occurrence has increased since its first description in 2010 (Cergole-Novella *et al.*, 2010). CTX-M-15 is the predominant ESBL among *Enterobacteriaceae* in Rio de Janeiro (Peirano *et al.*, 2011, Seki *et al.*, 2013).

Here, we have investigated the *bla*<sub>CTX-M-15</sub> gene presence in *E. coli* isolates causing urinary tract infections (UTI) in Rio de Janeiro. Clonal and phylogroups diversity was determined, as well as the ability of MALDI-TOF MS to detect ESBL in CTX-M-15-producing *E. coli* strains.

Thirty-two non-duplicated *E. coli* isolates were recovered from urine of outpatients assisted from June to October 2014 at a public hospital located in the west zone of Rio de Janeiro, Brazil. Bacterial identification was performed by using the Vitek2 automated system (bioMérieux, France) and conventional biochemical tests. Biochemical tests included glucose/lactose fermentation tests and H<sub>2</sub>S production in triple sugar iron agar media; motility and indole production in sulfide indole motility medium; citrate utilization; lysine/ornithine decarboxylation; urease test and culture in eosin methylene blue agar medium (Becton,

Dickinson, and Company, Sparks, MD). Susceptibility to twelve drugs (amikacin, cefotaxime, ceftazidime, ceftriaxone, cefepime, cephalothin, ciprofloxacin, gentamicin, imipenem, nalidixic acid, norfloxacin and trimethoprim-sulfamethoxazole) was determined by disk-diffusion test according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2016). *E. coli* ATCC 25922 was used as control. ESBL production was investigated by double-disk (DD) synergy test as previously proposed by Jarlier *et al.* (1998), with modifications, by placing disks of ceftazidime, cefepime, cefotaxime and aztreonam at distances of 20 mm (center to center) around an amoxicillin-clavulanic acid disk placed on the center agar surface. All *E. coli* isolates were submitted for detection of *bla*<sub>CTX-M-15</sub> gene by conventional PCR and sequencing using primers and conditions previously reported (Mulvey *et al.*, 2003). Evaluation of chromosomal polymorphisms was performed by pulsed-field gel electrophoresis (PFGE) of isolates by a previously reported method (Ribot *et al.*, 2006), with modifications. DNA was digested with *Xba*I (Boehringer Mannheim Biochemicals, Indianapolis, Ind). The restriction fragments were separated by PFGE in 1% (w/v) agarose gels in a CHEF DRIII system (Bio-Rad, Hercules, CA, USA). The banding patterns were interpreted with BioNumerics software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium) by using the Dice index and the unweighted pair group method with arithmetic averages. For phylogenetic analysis, DNA samples were subjected to PCR amplification for three markers: *chuA* gene, *yjaA* gene and TSPE4.C2 (Clermont *et al.*, 2000). Phylogenetic groups (A, B1, B2 and D) were determined based on presence (+)/absence (-) of the genes or DNA fragment studied. Through Clermont dichotomous tree,

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## Corresponding author:

Flávia Lúcia Piffano Costa Pellegrino.  
E-mail: flpellegrino@hotmail.com

the *chuA* gene presence separates B2 and D (+) of A and B1 (-) groups; *yjaA* gene presence separates B2 (+) of D (-) group, and TSPE4.C2 separates B1 (+) of A (-) phylogroup. Interpretation was performed according to Clermont *et al.* (2013) criteria [14]: phylogroup A (*chuA* -, *yjaA* -, TspE4.C2 -; or *chuA* -, *yjaA* +, TspE4.C2 -), phylogroup B1 (*chuA* -, *yjaA* -, TspE4.C2 +), phylogroup B2 (*chuA* +, *yjaA* +, TspE4.C2 +; or *chuA* +, *yjaA* +, TspE4.C2 -; or *chuA* +, *yjaA* -, TspE4.C2 +) and phylogroup D (*chuA* +, *yjaA* -, TspE4.C2 -; or *chuA* +, *yjaA* -, TspE4.C2 +). MALDI-TOF MS efficiency for rapid ESBL detection in the CTX-M-15-producing *E. coli* isolates was verified by observation of the cefotaxime's hydrolysis, as well as its inhibition by clavulanic acid based on protocols previously described by Sparbier *et al.*, 2012, with modifications. MALDI-TOF MS assays were performed by incubating fresh *E. coli* inoculums grown on Nutrient and Eosin Methylene Blue agar plates for 18 h at 37°C (Oxoid) in solutions with or without cefotaxime (Sigma-Aldrich, Germany; 10 µg/mL in H<sub>2</sub>O) and with cefotaxime plus clavulanic acid (10 µg/mL in in H<sub>2</sub>O) for 3 h at 37°C. After centrifugation (3 min at 13,000 xg at room temperature), 0.3-µL aliquots of fresh supernatant were overlaid with 0.3 µL of α-cyano-4-hydroxycinnamic acid matrix solution (HCCA Bruker Daltonics, Bremen, Germany), spotted onto a 96-spot polished-steel MALDI target plate. Mass spectra were obtained by using Bruker Daltonics Microflex (Bremen, Germany) equipment at Proteomic Platform (PDTIS-IOC, Fiocruz) operating in positive linear ion mode in mass range of 100 Da to 1,000 Da with the maximum laser frequency. HCCA peak at 379 Da was used for calibration. Parameters and the peaks interpretation were performed according to Sparbier *et al.* (2012). Conventional phenotypic tests confirmed the *E. coli* species. By disk-diffusion test, all bacterial isolates were susceptible to imipenem (32; 100%), only two were susceptible to all antimicrobial agents tested. The highest resistance rates were observed for nalidixic acid (13; 41%), norfloxacin and ciprofloxacin (12; 37%) and trimethoprim-sulfamethoxazole (11; 34%). The lowest resistance rate was detected for ceftazidime (3; 9%). DD synergy test for ESBL production was positive to five *E. coli* isolates resistant to cefotaxime (16%). *bla*<sub>CTX-M-15</sub> gene was detected, by PCR, in the five ESBL-producing *E. coli* isolates; DNA sequencing confirmed the CTX-M-15 variant. *E. coli* isolates not-carrying *bla*<sub>CTX-M-15</sub> gene were susceptible to cefotaxime and negative at DD synergy test. Thirty-two dis-

tinct PFGE patterns were observed, showing a high clonal diversity among *E. coli* isolates, including the CTX-M-15-producing *E. coli* strains (Table 1). *E. coli* isolates belonged to three Clermont phylogroups, most of them to B2: A (7, 22%), B1 (5, 16%) and B2 (20, 62%).

No influence of culture medium used to *E. coli* growth was observed on MALDI results. Cefotaxime peaks and its respective degradation products were labeled. Cefotaxime molecular peak ([M+H]<sup>+</sup> 456.1 Da) was detectable in the spectrum derived from *E. coli* ATCC 25922 control strain. 414.1 Da peak corresponded to the deacetylated form due to spontaneous hydrolysis of cefotaxime and 396.1 Da peak to the non-hydrolyzed-cefotaxime form, corresponding to elimination of the acetyl group of cefotaxime. As expected, in the cefotaxime-susceptible *E. coli* isolates, 456.1 Da peak was visualized but disappeared in the cefotaxime-resistant isolates. In the bacterial suspensions treated with ESBL inhibitor (clavulanic acid), 369.9, 445.0 and 460.9 Da peaks, corresponding the cefotaxime's hydrolysis products, disappeared or decreased, while 456.1 Da (cefotaxime molecular peak) and 396.1 Da (non-hydrolyzed-cefotaxime form) peaks were detected again. Mass spectra obtained by MALDI-TOF MS for *E. coli* ATCC 25922, for one *E. coli* isolate not carrying *bla*<sub>CTX-M-15</sub> gene (112/09) and for one CTX-M-15-producing *E. coli* strain (169/09) are shown in Figure 1. In *E. coli* ATCC 25922, cefotaxime (456.1 Da) and cefotaxime's acetylated form (414.1 Da) peaks were visualized (A). A similar mass spectra pattern was observed to the *E. coli* strain not carrying the *bla*<sub>CTX-M-15</sub> gene (112/09) (B). In CTX-M-15-producing *E. coli* strain (169/09), the three peaks referred to cefotaxime's hydrolysis (369.9, 445.0 and 460.9 Da) and the increase of the 414.1 Da peak were detected (C). In cefotaxime's hydrolysis inhibition reactions by clavulanic acid, 369.9 Da, 445.0 Da, and 460.9 Da peaks were not detected in the CTX-M-15-producing 169/09 strain, but 456.1 Da and 396.1 Da peaks reappeared, confirming ESBL activity's inhibition (D) (Figure 1).

The cefotaximase "CTX-M" is an Ambler class A ESBL enzyme, typical among *Enterobacteriaceae*, with powerful ability to hydrolyze cefotaxime (Tzouveleki *et al.*, 2000), preferable to other cephalosporins such as ceftazidime, for example (Poirel *et al.*, 2001).

In the present study, 32 *E. coli* isolates causing UTI in outpatients in Rio de Janeiro were analyzed as to antimicrobial susceptibility and molecular epidemiology. Resistance

**Table 1** - Key characteristics of the five uropathogenic *Escherichia coli* strains carrying the *bla*<sub>CTX-M-15</sub> gene.

Isolate code	Isolation date	Resistance pattern	DD test	<i>bla</i> <sub>CTX-M-15</sub> PCR	MALDI-TOF MS	PFGE genotype	Phylogroup
283/06	05/06/2014	AMI CAZ CEF CIP CFT CTX GEN NAL NOR SUT	+	+	CTX R/CTX+CLA S	A	B2
169/09	05/09/2014	AMI CEF CIP CFT CTX GEN NAL NOR SUT	+	+	CTX R/CTX+CLA S	B	B2
225/09	07/09/2014	GEN CFT CTX	+	+	CTX R/CTX+CLA S	C	A
119/09	07/09/2014	AMI CAZ CEF CIP CFT CTX GEN NAL NOR SUT	+	+	CTX R/CTX+CLA S	D	B2
83/09	22/09/2014	CEF CIP CFT CTX NAL NOR SUT	+	+	CTX R/CTX+CLA S	E	A

Drugs tested: AMI: amikacin; CAZ: Ceftazidime; CEP: cefepime; CIP: ciprofloxacin; CFT: Ceftriaxone; CTX: cefotaxime; GEN: gentamicin; IMI: imipenem; NAL: Nalidixic acid; NOR: norfloxacin; SUT: trimethoprim-sulfamethoxazole; CLA: clavulanic acid.

R: Resistant; S: Susceptible.

ESBL: Extended-spectrum beta-lactamase.

DD test: double-disk synergy test for ESBL production detection; +: positive.

PFGE: pulsed-field gel electrophoresis.

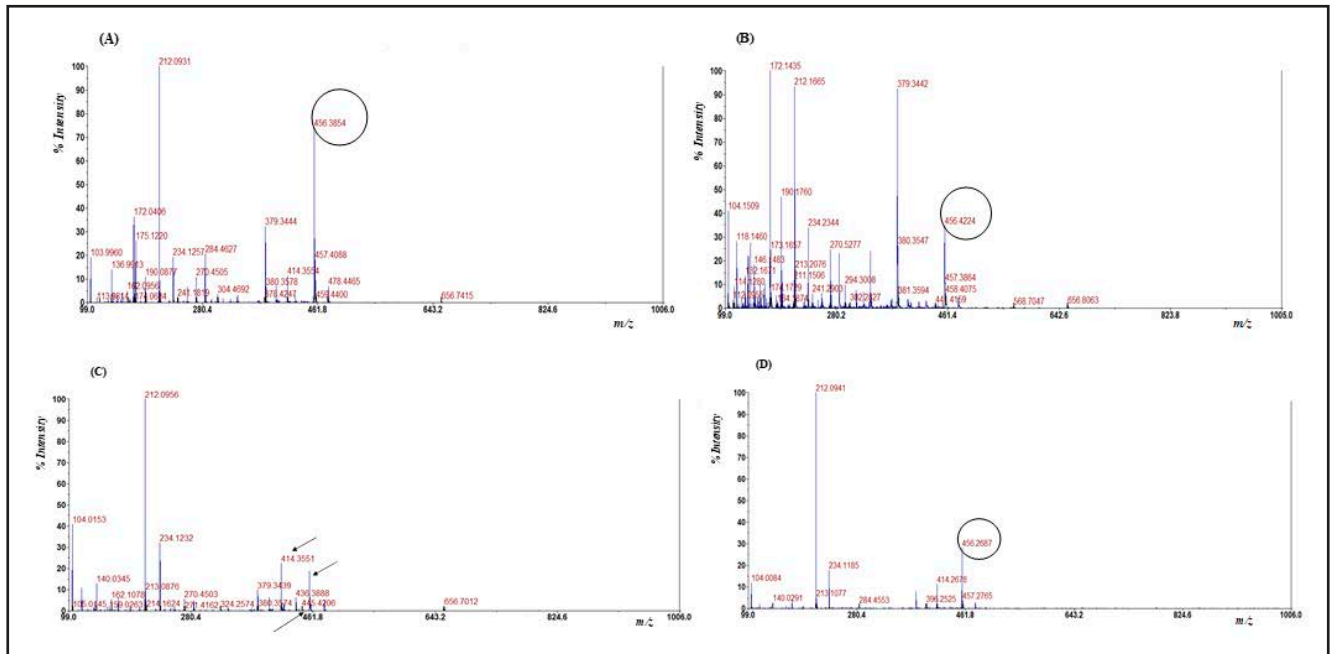


Figure 1 - Mass spectra obtained by MALDI-TOF MS analysis after incubation of cefotaxime solution with *Escherichia coli* ATCC 25922 control strain (A), *E. coli* strain CTX-M-15-non-producer (cefotaxime-susceptible, DD synergy test negative for ESBL, and not-carrying *bla*<sub>CTX-M-15</sub> gene) (112/09 strain) (B) and *E. coli* strain CTX-M-15-producer carrying the *bla*<sub>CTX-M-15</sub> gene (169/09 strain) (C). Circles represent the matrix cefotaxime's peak (456 Da). Arrows represent matrix peaks corresponding to cefotaxime's hydrolyzed forms generated by the CTX-M-15 ESBL action. Inhibition of cefotaxime's hydrolysis observed in the *E. coli* strain CTX-M-15-producer (169/09 strain) in the clavulanic acid presence was interpreted by the reappearance of the cefotaxime's peak (456 Da) in the mass spectra (D).

rates varied from 9% (ceftazidime) to 41% (nalidixic acid). DD synergy test to ESBL production was positive for five *E. coli* isolates, all resistant to cefotaxime (16%), suggesting CTX-M activity. *bla*<sub>CTX-M-15</sub> gene was detected in the five ESBL-producing *E. coli* isolates (Table 1), identified by sequencing as the CTX-M-15 variant. The CTX-M-15-producing *E. coli* prevalence (16%) was higher than that determined by another local study when considering the *E. coli* isolates recovered from urine (13%) (Peirano *et al.*, 2011). Eight *E. coli* phylogroups (A, B1, B2, C, D, E, F and *Escherichia* crypt clade I) have been described so far (Herzer *et al.*, 1990). Virulent extra-intestinal *E. coli* strains, such as those causing UTI, mainly belong to phylogroup B2 and, in lesser range, to group D; commensal strains belong to group A (Picard *et al.*, 1999). Our phylogenetic analyses revealed that most *E. coli* isolates belonged to B2 phylogroup (62%), as reported by da-Silva and collaborators whose phylogroup B2 was the most frequent (83% in men and 43% in women) among *E. coli* isolates causing community-acquired UTI in Rio de Janeiro (da-Silva *et al.*, 2017). Phylogroups determination by Clermont scheme is a simple, rapid and efficient method not just for determining the most prevalent *E. coli* phylogroups causing UTI, but also for recognizing, in the *E. coli* population, potential pathogenic *E. coli* strains (Clermont *et al.*, 2000). One PFGE "dominant" clone belonging to phylogroup A was described in 2011 among *E. coli* isolates from Rio de Janeiro, although not all were recovered from urine (Peirano *et al.*, 2000). Here, 32 distinct genotypes were generated by PFGE, showing the wide variety of clones among *E. coli* isolates of the study. PFGE results point out that, among *E. coli* strains causing UTI in outpatients, there must neces-

sarily be no relationship between resistant strains and predominant PFGE clones or phylogroups. MALDI-TOF MS has been used to presume bacterial resistance against different antibiotics; the most tested agents are beta-lactams, mainly carbapenems (Burckhardt *et al.*, 2011). MALDI performance for beta-lactamases detection was evaluated in previous Brazilian studies (Carvalhoes *et al.*, 2013; Carvalhoes *et al.*, 2014). In the present work, for the first time, we have evaluated the MALDI-TOF MS ability to detect ESBL in CTX-M-15-producing uropathogenic *E. coli* isolates. MALDI-TOF MS proved to be efficient for ESBL detection, being clearly possible to observe rapid cefotaxime hydrolysis as well as its inhibition by clavulanic acid, a characteristic behavior of CTX-M activity. MALDI-TOF MS can be a useful tool for ESBL-producing *E. coli* isolates screening in microbiological diagnostic of UTI.

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