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Escherichia coli

Detection of β-lactamase genes $^{bla}_{\text{TEM}}$, $^{bla}_{\text{SHV}}$ and $^{bla}_{\text{CTX-M}}$ in community isolates of *Escherichia coli*

Detección de los genes de β-lactamasas $^{bla}_{\text{TEM}}$, $^{bla}_{\text{SHV}}$ y $^{bla}_{\text{CTX-M}}$ en aislamientos de *Escherichia coli* comunitarios

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Abstract: Globally, resistance to antibiotics is a public health problem, both in the hospital and in the community environment. The production of β-lactamases is the main mechanism of resistance in enterobacteria and usually the cause of resistance are the enzymes to the families TEM, SHV and CTX-M. The principal aim of this study was to detect - β-lactamase genes $^{bla}_{\text{TEM}}$, $^{bla}_{\text{SHV}}$ and $^{bla}_{\text{CTX-M}}$ in community strains of ESBL-producing *Escherichia coli* isolated from urine cultures of patients attended in the Laboratorio Clínico Popular of the Universidad de San Carlos de Guatemala in 2016. At least, one of the genes was detected in 90% of the 79 isolates and in 53.2% of the isolates the three genes were detected. The frequency was 57% for $^{bla}_{\text{CTX-M}}$, 84% for $^{bla}_{\text{SHV}}$ and 85% for $^{bla}_{\text{TEM}}$.The detection of the genes coding for TEM-1, SHV-11, CTX-M15 and CTX-M55 represent the first molecular characterization of ESBL-producing *E. coli* isolates in Guatemala and it is important to understand the spread of these strains at the community environment. The ESBL-producing *E. coli* isolates showed high resistance to ciprofloxacin and trimethoprim sulfamethoxazole (78%) and low resistance levels for fosfomycin (2.5%) and nitrofurantoin (7.6%). The 11.39% of the strains showed resistance to a group of non-beta-lactam antibiotics. It is important to establish an active surveillance for these antibiotics in community strains because they are the first line treatment for ESBL-producing strains.

Keywords: extended spectrum β-lactamases (ESBL), community isolates, *Escherichia coli*, TEM, SHV, CTX - M.

Resumen: A nivel mundial la resistencia a los antibióticos es un problema de salud pública, tanto en el ámbito hospitalario como en el comunitario. La producción de β-lactamasas es el principal mecanismo de resistencia en enterobacterias y la mayoría de enzimas responsables pertenecen a las familias TEM, SHV y CTX-M. El objetivo de este estudio fue detectar los genes de β-lactamasas $^{bla}_{\text{TEM}}$, $^{bla}_{\text{SHV}}$ and $^{bla}_{\text{CTX-M}}$ en cepas comunitarias de *Escherichia coli* productoras de BLEE aisladas de urocultivos de pacientes que acudieron al Laboratorio Clínico Popular de la Universidad de San Carlos de Guatemala en el año 2016. Se detectó la presencia de al menos uno de los genes en el 90% de los 79 aislamientos y un 53.2% presentó los tres genes. La frecuencia fue de 57% para $^{bla}_{\text{CTX-M}}$, 84% para $^{bla}_{\text{SHV}}$ y 85% para $^{bla}_{\text{TEM}}$. La detección de los genes codificadores de las enzimas TEM-1, SHV-11, CTX-M15 y CTX-M55

corresponde a la primera caracterización molecular de aislamientos de *E. coli* productoras de BLEE en Guatemala y son importantes para entender su propagación en el ámbito comunitario. Los aislamientos de *E. coli* productoras de BLEE mostraron alta resistencia a ciprofloxacina y trimetoprim sulfametoxazol (78%) y bajos niveles de resistencia para fosfomicina (2.5%) y nitrofurantoina (7.6%). El 11.39% de las cepas presentó resistencia a un grupo de antibióticos no betalactámicos. Es importante establecer una vigilancia activa para la resistencia de estos antibióticos en cepas comunitarias ya que son la primera opción de tratamiento para cepas productoras de BLEE.
Palabras clave: β-lactamasas de espectro extendido (BLEE), aislamientos comunitarios, *Escherichia coli*, TEM, SHV, CTX - M.

Introduction

The production of β-lactamase is the main mechanism by which resistance in Gram-negative bacilli is produced; especially because genes encoding these enzymes are found in plasmids, which transmit to other bacteria and simultaneously encode genes of resistance for different antibiotics (Geser, Stephan, & Hächler, 2012; Mulvey et al., 2004; Paterson, et al., 2003).

Extended Spectrum β-lactamases (ESBL) are enzymes that hydrolyze penicillins, aztreonam, and cephalosporin including an oxyimino side chain (first, second and third generation). However, they have no effect on cephamycins nor carbapenems, and are inhibited by clavulanic acid and other inhibitors such as sulbactam or tazobactam (Jeong et al., 2004; Kiratisin, Apisarnthanarak, Laesripa, & Saifon, 2008; Tankhiwale, Jalgaonkar, Ahamad, & Hassani, 2004).

The first ESBL was isolated in Germany in 1983 and 200 more types have been characterized worldwide ever since. More disseminated ESBL come from β-lactamases type TEM, SHV and CTX-M through point mutations in ^{bla}TEM, ^{bla}SHV y ^{bla}CTX-M genes that disrupt the amino acid sequence of the enzyme active site and increase its hydrolysis capacity for certain substrates, like cephalosporins of third and fourth generation (Mulvey et al., 2004; Paterson, et al., 2003).

For quite a long time, infections caused by ESBL-producing strains were linked to different hospital-originated factors that would increase any probability of acquiring an infection, e.g., long hospital stays in intensive care units, long term invasive medical procedures, (central catheter, urinary stent, urotracheal tubes, among others), hemodialysis and malnutrition. Recently, different studies on community isolates of enterobacterium have shown that outpatients can act as reservoirs of ESBL producing strains, helping to its dissemination, when having recurrent urinary infections, being previously admitted to hospitals or having chronic kidney, pulmonary, liver, cardiovascular disease or diabetes (Paterson & Bonomo, 2005; Ben-Ami et-al., 2006).

Diverse studies that have molecularly characterized ESBL in hospital and community isolates determined a high

frequency of β-lactamases derived from TEM and SHV. However, there has been a frequency escalation of ESBL-producing strains of CTX-M-type, which has been connected to the use of ceftriaxone globally. These findings should be of concern due to a global tendency of acquired infections outbreak in the community caused by ESBL-producing strains of CTX-M-type, whose dissemination is associated to inefficient policies on antibiotics at a hospital and outpatient level (Blanco et al., 2013; Mendonça, Leitão, Manageiro, Ferreira, & Caniça, 2007; Paterson & Bonomo, 2005).

In order to determine ESBL prevalence in Guatemala, studies have been carried out in different care centers (hospitals) and isolates from the community, reporting prevalences of 31.9% and 27% respectively (Alvarado & Mejía, 2014; Gordillo, Mejia, & Matheu, 2014). Nevertheless, no data hasn't been found regarding the specific ESBL type produced by these enterobacterium, especially in community isolates.

The objective of this study was to detect β-lactamases genes ^{bla}TEM, ^{bla}SHV y ^{bla}CTX-M in community isolates of Escherichia coli based on urine culture of patients receiving care at Laboratorio Clínico Popular (LABOCLIP) of the University of San Carlos de Guatemala during the first four months of 2016. Polymerase chain reaction and bidirectional Sanger sequencing were used to determine such genes. The obtained results represent the first national data on molecular epidemiology of ESBL-producing E coli strains being present at a community level around the country, which is important for both monitoring and surveillance of antimicrobial resistance.

Materials y methods

Selection of strains

From January to April 2016, 94 community isolates of ESBL-producing *E. coli* based on urine culture were gathered at the microbiology area in Laboratorio Clínico Popular (LABOCLIP). The resistance mechanism was detected by using the Kirby-Bauer disk method containing cefotaxime (30 μg) (OXOID, Hampshire UK), ceftazidime (30 μg) (OXOID, Hampshire UK), and amoxicillin/clavulanic acid (20/10 μg) (OXOID, Hampshire UK). Fifteen urine cultures were excluded due to the absence of bacterial growth besides the extracted DNA quality and quantity not being appropriate after two repetitions.

Enrichment and DNA extraction

E. coli strains were inoculated in 5 ml of LB (Luria-Berthani) broth for 48 h at 37° for its enrichment. Subsequently, 400 μL of culture was taken in order to extract DNA through the Wizard® Genomic DNA Purification Kit, according to the directions by the manufacturing company (Promega, Madison USA).

Polymerase chain reaction (PCR) of β-lactamases genes

Sanger sequencing

The obtained PCR products for five strains were sequenced by using the Sanger bidirectional method at the company Macrogen, USA (Maryland, USA). The results were then analyzed on the Geneious software R8.8.1.8, and the sequences were converted to proteins to be analyzed on the software CLC sequence viewer 8.0 Qiagen®.

Analysis of results

Frequencies, mean and proportions, were used in order to describe: sex and age of the patients coming from isolates, presence or absence of β-lactamases genes, and resistance or susceptibility to antibiotics. A research matter was to find differences between the resistance to non-β-lactam antibiotics of the isolates from patients with or without any urinary tract disease history. Both descriptive statistics of the isolates and resistance profiles were analyzed on Microsoft Excel. The comparisons between groups were made with the Chi^2 test through a computer tool called OpenEpi version 3.01, and a p value $\leq .05$.

Results

An 87% (66/79) of isolates were from urine cultures in women. The mean age was 57 years old, interquartile rates (IQR= 26 – 67 years of age) for all patients, and 31.6% (25/79) presented history of recurrent urinary tract infection (UTI).

By means of the polymerase chain reaction technique (PCR), it was possible to analyze 79 ESBL-producing strains and to determine the presence of genes $^{bla}_{\text{TEM}}$, $^{bla}_{\text{SHV}}$ y $^{bla}_{\text{CTX-M}}$ (Figure 1). The presence of at least one of the analyzed genes was detected in 71 (90%) isolates.

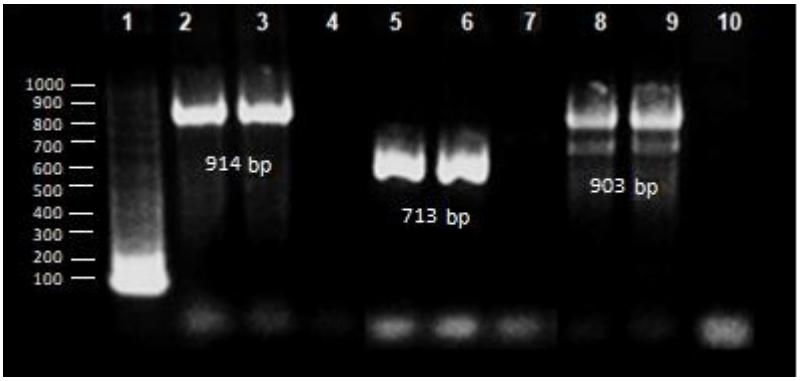

Figure 1.

PCR products with 2% of agarose gel. Columns 2 and 3 $^{bla}_{\text{TEM}}$, columns 5 and 6 $^{bla}_{\text{SHV}}$, columns 8 and 9 $^{bla}_{\text{CTX-M}}$, columns 4, 7 and 10 PCR reagent blank. Column 1 Molecular weight marker 100 bp.

More than one β-lactamase was found in 82.3% of strains, and in more than half (53.2%), the presence of three genes was spotted. Enzyme-encoding genes of TEM and SHV type were solely detected in 3.8% of isolates as opposed to the CTX-M type, which

were jointly detected along with TEM and/or SHV enzymes. However, the genes in discussion were absent in 10.1% of isolates (Table 1).

Table 1.
Frequency of detected β-lactamase genes

β-lactamase genes	Isolates (N= 79)	Isolates %
bla_{TEM}, bla_{SHV} and bla CTX-M	42	53.2
bla_{SHV}, bla_{CTX-M}	2	2.5
bla_{TEM}, bla_{CTX-M}	1	1.3
bla_{TEM}, bla_{SHV}	20	25.3
bla_{TEM}	3	3.8
bla_{SHV}	3	3.8
Negative (other ESBL)	8	10.1

Prevalence of blaTEM, blaSHV and blaCTX-M genes detected in five strains, previously analyzed, helped to determine the specific enzyme causing the resistance mechanism (Figures 2, 3 and 4). The specific types of obtained β-lactamase can be seen on Table 2.

Table 2.
Tipo de β- lactamasa determinado por secuenciación de Sanger bidireccional de productos de PCR

Analyzed strain	β-lactamase type
ECO-57	CTX-M15, SHV-11
ECO-96	CTX-M55
ECO-97	TEM-1
ECO-109	CTX-M15, SHV-11 and TEM-1
ECO-112	SHV-11 and TEM-1

Figure 2.

Electropherogram of PCR product for *bla*$_{\text{TEM}}$. The alignment between the obtained sequence for the ECO-109 sample through Sanger bidirectional sequencing and the consensus sequence for TEM-1 enzyme (KR632746) can be seen. (A=adenine/red, C=cytosine/blue, T=thymine/green and G=guanine/yellow) Software: Geneious R8 8.1.8

Figura 3.

Electropherogram of PCR product for *bla*$_{\text{SHV}}$. The alignment between the obtained sequence for the ECO-109 sample through Sanger bidirectional sequencing and the consensus sequence for SHV-11 enzyme (KR632750) can be observed. (A=adenine/red, C=cytosine/blue, T=thymine/green and G=guanine/yellow) Software: Geneious R8 8.1.8 Import table

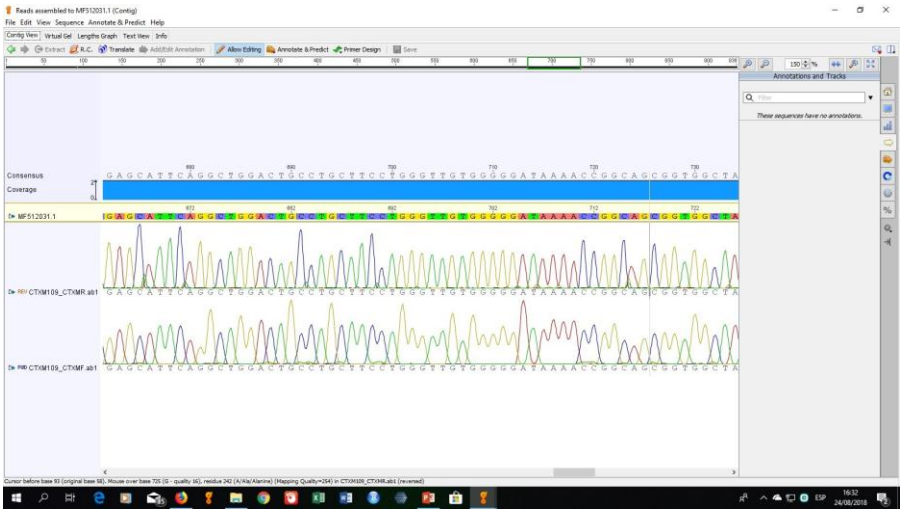

Figure 4.

Electropherogram of PCR product for bla_{CTX-M}. The alignment between the obtained sequence for the ECO-M109 sample through Sanger bidirectional sequencing and the consensus sequence for CTX-M15 enzyme (MF512031.1) can be observed. (A=adenine/red, C=cytosine/blue, T=thymine/green and G=guanine/yellow) Software: Geneious R8 8.1.8

Table 3 shows the resistance profile of the evaluated strains as opposed to non-β-lactam antibiotics, included in the antibiogram routine testing at LABOCLIP. The highest resistance percentages correspond to ciprofloxacin and trimethoprim-sulfamethoxazole (both 62/79). On the contrary, resistance resulted low for fosfomycin (2/79) and nitrofurantoin (6/79).

Table 3.

Resistance profile to non-β-lactam antimicrobials of analyzed *E. coli* isolates

Antibiotic	Resistant α		Susceptible	
Fosfomycin β	2	2.5%	68	86.1%
Nitrofurantoin	6	7.6%	73	92.4%
Ciprofloxacin	62	78.5%	17	21.5%
Amoxicillin/Clavulanic acid	34	43.0%	45	57.0%
Trimethoprim-sulfamethoxazole	62	78.5%	17	21.5%

α It includes antibiogram resistant and intermediate results β Nine isolates were not evaluated for fosfomycin

Resistance to non-β-lactam antibiotic was found in 11.39% (9/79) of the analyzed strains, such resistance turned out to be diverse for the most part. A 59.49% (47/79) was resistant to ciprofloxacin combined with trimethoprim-sulfamethoxazole, resulting in the most common resistance profile. Moreover, 29.11% of strains resistant to both antibiotics also showed resistance to amoxicillin/clavulanic acid combination.

When patients with recurrent UTI history and without this background were compared (p=.84, p=.17 and p=.55, respectively),

no significant difference was found between the resistance to ciprofloxacin, trimethoprim-sulfamethoxazole and amoxicillin/clavulanic acid.

Discussion

LABOCLIP is a laboratory school that daily receives approximately 300 outpatients, most of whom have limited resources. In 2006, ccording to the records from the Clinical Microbiology area, 36% positive ESBL *E. coli* was isolated in 36% of urine cultures, which made this bacteria the most common pathogen associated with urinary tract infections with 93%.

An alarming increase of ESBL-producing strains of community isolates has been reported worldwide, especially in pathogens from the Enterobacteriaceae family (Fan et al., 2014; Pitout, Nordmann, Laupland & Poirel, 2005; Shaikh, Fatima, Shakil, & Rizvi, 2015; Tumbarello et al., 2006). Both detection and characterization of these isolates are extremely important in order to determine therapy, as well as their control and prevention. For that reason, detection of $^{bla}_{\text{TEM}}$, $^{bla}_{\text{SHV}}$ y $^{bla}_{\text{CTX-M}}$ genes in ESBL-producing community isolates of *E. coli* isolated from urine cultures was proposed.

At least one of the analyzed genes was present in 89.8% of isolates (71/79). The most common genes were $^{bla}_{\text{TEM}}$ and $^{bla}_{\text{SHV}}$ with 84 and 85% respectively. However, one of the main ESBL characterization issues is that they are usually associated with broad spectrum β-lactamases (BSBL), especially with TEM and SHV types, from which they derive. This suggests the necessity to identify the specific enzyme type to be able to distinguish them among others (Bradford, 2001; Jacoby & Munoz-Prince, 2005; Paterson & Bonomo, 2005).

On the contrary, detection of $^{bla}_{\text{CTX-M}}$ gene in ESBL-producing isolates is sufficient evidence to explain the resistant phenotype. This means that in 57% of the analyzed isolates, CTX-M enzyme is responsible for the resulted resistance pattern, regardless of the presence of TEM and SHV enzymes, whose genes were also spotted. This percentage agrees with previous studies on *E. coli* community strains worldwide, where 40 and 70% of CTX-M-type ESBL has been reported (Brigante et al., 2005; Rodriguez-Villalobos et al., 2011; Shaikh et al., 2015).

In 10% of isolates showing absence of the analyzed genes, it is indispensable to evaluate the presence of encoding genes for other enzyme types, such as GES, BES, TLA, SFO and IBC, which present extended spectrum and have been described in enterobacteria (Bradford, 2001; Paterson & Bonomo, 2005; Shaikh et al., 2015).

In order to determine the specific type of present enzymes, five strains were selected for sequencing of PCR products. The strain

encoded as ECO-109 was selected because it showed a multiresistance profile (resistant to all evaluated non-β-lactam antibiotics), whereas strains ECO-57, ECO-96, ECO-97 and ECO-112 were randomly selected. The obtained sequencing results show that the present genes correspond to enzymes TEM-1, SHV-11, CTX-M15 and CTX-M55.

TEM-1 enzyme is a broad spectrum β-lactamase identified in 1965 for the first time, which is responsible of approximately 90% of plasmid-mediated ampicillin resistance in *E. coli* (Bradford, 2001; Paterson & Bonomo, 2005). This enzyme was detected in strains ECO-97, ECO-109 and ECO-112, which is certainly why in these three isolates the resulted ESBL phenotype do not derive from TEM-type enzymes.

SHV-11 enzyme, whose gene was detected in the three sequenced strains (ECO-57, ECO-109, and ECO-112), was described in 1997. Unlike SHV-1 enzyme (first enzyme to be describe within this group), SHV-11 shows a glutamine at position 35 instead of a leucine. This change is found far-off from the enzyme active site, reason why the hydrolysis spectrum of SHV-11 is the same for SHV-1 (Nüesch-Inderbinen, Kayser, & Hächler, 1997). Therefore, this enzyme presence, just like TEM-Q presence, suggests that the determined ESBL phenotype in strains is caused by another enzyme.

CTX-M15 enzyme, whose gene was detected in two isolates from this study, was first isolated in India and constitutes the most disseminated CTX-M-type enzyme around the globe. It is part of the "CTX-M pandemic", associated with urinary tract infections (UTI) and community-acquired bacteremias (Coque et al., 2008; Hijazi, Fawzi, Ali, & Abd El Galil, 2016; Moubareck et al., 2005). The $^{bla}_{CTX\text{-}M15}$ gene has been found in highly efficient mobile elements, such as plasmids IncFI, IncFII, IncHI2, IncI, IncN, IncP-1-a, IncL/M e IncA/C; and sequences ISEcp1 and ISCR1 (Brigante et al., 2005; Peirano & Pitout, 2010). Recent surveillance studies inform report high percentages of CTX-M15-producing *E. coli* isolates in Europe, Asia and South America (Hijazi et al., 2016; Lau et al., 2008; Shi et al., 2015).

Studies on molecular epidemiology with MLST technique (multilocus sequencing typing) suggest that the fast CTX-M15-producing *E. coli* spreading worldwide has happened as a result from the ST131 clone. This highly virulent clone also has resistance genes, such as $^{bla}_{TEM\text{-}1}$, $^{bla}_{OXA\text{-}1}$, $^{aaa(6´)\text{-}Ib\text{-}cr,\ tetA\ y\ aaa(3)\text{-}II}$, contained in class 1 integrons or associated to IncFII plasmid (Machado et al., 2006; Peirano, Costello, & Pitout, 2010; Shi et al., 2015). Future cloning studies in ESBL-producing *E. coli* strains could explain the high frequency of enzymes co-producing strains of type TEM-1 and CTX-M15 (54%), obtained in this study, as well as combined resistance to fluoroquinolones.

Enzymes *aaa(3)-II and aaa(6´)-Ib*-cr have been associated with CTX-M15-producing *E. coli* (Peirano & Pitout, 2010; Xiao & Hu,

2012). These enzymes modify aminoglycosides and inactivate them. It was not possible in this study to determine the resistance to this antibiotic group, since they are not put through a routine evaluation in the antibiogram used for Gram-negatives at LABOCLIP. ECO-109 strain was resistant to the evaluated routine antibiotics, which is why it encountered two aminoglycosides and presented resistance to gentamicin and susceptibility to amikacin. The presence of these two resistance genes could be analyzed in this particular strain in order to confirm the resulted phenotype.

The enzyme *aaa(6´)-Ib-cr* also has acetylation capacity, and therefore inactive fluoroquinolones presenting free amino nitrogen on the piperazine ring (norfloxacine and ciprofloxacin) (Park, Robicsek, Jacoby, Sahm, & Hooper, 2006; Peirano & Pitout, 2010). This enzyme combined presence with $^{bla}_{CTX\text{-}M15}$ would then explain the resulted high resistance to fluoroquinolones (ciprofloxacin) by the analyzed strains (78.5%), even though, presence of other genes should be evaluated, such as gyrA, parC (chromosomal), qnrA, qrnB and anrS (plasmid), also associated to these antibiotic resistance (Hernández, 2010; Jacoby et al., 2006; Peirano et al., 2010; Peirano & Pitout, 2010). High fluoroquinolone resistance range detected in ESBL-producing strains, agrees with prevalence reported in other studies, like Rodríguez-Baño´s et al., who found 77% of fluoroquinolone resistance in ESBL-producing strains (Coque et al., 2008; García-Gómez, Guio, Hernández, Vilar & Pijoán, 2015; Rodríguez-Baño et al., 2004; Rodriguez-Villalobos et al., 2011).

CTX-M55 enzyme belongs to the CTX-M1 subgroup, just like CTX-M15 type, from which it differentiates only by having a valine instead of an alanine at position 77, reason why it is believed it derived from this enzyme. It was first described in Thailand and structural studies have shown that it is associated to the presence of ISEcp1, just like CTX-M15 (Kiratisin, et al., 2007). It is the second most frequent CTX-M-type enzyme and it has been isolated in humans and animals (Kim et al., 2017). The strain ECO-96, which showed presence of this enzyme, was resistant to fosfomycin, some interesting data, especially because the combined production of CTX-M55 enzyme and fosA3 gene in *E. coli* isolates in Brazil and France has been described (Cunha, Lincopan, Cerdeira, & Esposito, 2017; Fernandes, Sellera, Moura, Souza, & Lincopan, 2018; Lupo, Saras, Madec, & Haenni, 2018).

Both nitrofurantoin and fosfomycin were the antibiotics that presented presented low resistance range (7.6 and 2.5% respectively). However, these ranges are high compared to previous international reports of 0.3% of strains resistant to fosfomycin and 1.5% resistant to nitrofurantoin in community isolates. High resistance levels to antibiotics are associated to ineffective policies for antibiotic stewardship in hospitals and outpatient care, sanitary deficiency, presence and collective spread of resistance genes to various antibiotic types, and colonization of non-identified animal

and human reservoirs (Dash, Al-zarouni, Al-kous, & Shehhi, 2008; Zhanel et al., 2006).

The great amount of multi-resistant organisms in outpatients represents an emergent threat for public healthcare in the country, since it demonstrates resistant bacterial strain flow at a community level. Resistance to fluoroquinolone, trimethoprim, sulfamethoxazole and amoxicillin/clavulanic acid in ESBL-producing strains restrict therapy from using carbapenems, nitrofurantoin and fosfomycin as the first option for urinary tract infection treatment, cases originated within the community. This is alarming, since a frequency of 91% of strains carrying carbapenem resistance genes has been reported in hospital sectors (Velásquez Porta, 2016).

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