Detection of Multidrug-resistant Extended-spectrum Beta-lactamase-producing Enterobacteria from Community Infections in the city of Reynosa, Tamaulipas Mexico

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Abstract

Production of extended spectrum beta-lactamases (ESBL) is one of the main problems related to antimicrobial resistance worldwide, with the CTX-M, TEM and SHV types standing out as the most prevalent. These enzymes are usually related to plasmids which facilitates their horizontal genetic transmission. In the northeast region of Tamaulipas their clinical prevalence is unknown. Therefore, the aim of this work was to define the molecular epidemiology of ESBL-producing *Enterobacteriaceae* in clinical strains collected in Reynosa Tamaulipas, Mexico. A selection of 123 *Enterobacteriaceae* strains from different clinical patients were collected from August 2018 to December 2019. These strains were phenotypically identified by double disk synergy tests (DDST) and subsequently subjected to polymerase chain reaction for the detection and amplification of the bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$ and $bla_{CTX-M-8/25}$ genes. Lastly, antimicrobial resistance profiles were determined by plate diffusion method and their capacity to transfer this sort of resistance by conjugation was assessed. Our results showed a prevalence of 48.78% (60/123) of ESBL-producing enterobacteria, with the bla_{TEM} and $bla_{CTX-M-1}$ genes most commonly detected in 76.67% (46/60) and 58.33% (35/60), respectively. Additionally, a 68.33% (41/60) of these ESBL-producing *Enterobacteriaceae* were multidrug-resistant, while 51.67% (31/60) were able to transfer some genes related to ESBL production, being $bla_{CTX-M-1}$ the most common. This is the first study in the region that evaluates ESBL production in clinical *Enterobacteriaceae* strains, as well as the content of genes related to this phenotype and the ability to transfer this type of antimicrobial resistance.

Introduction

Antimicrobial resistance is currently a great challenge in the clinical environment, since it complicates the treatment, control, and prevention of a growing number of infections [1, 2, 3]. Infections related to multidrug-resistant bacteria (MDR) have led to the use of broad-spectrum antibiotics such as third and fourth generation cephalosporins and carbapenems, causing an increase on selective pressure that has favored the proliferation and dissemination of resistant strains [4, 5]. Extended-spectrum beta-lactamases (ESBL) producing *Enterobacteriaceae* are considered by the World Health Organization (WHO) as priority microorganisms for the development of new antibiotics, as well as for microbial resistance monitoring [6]. ESBL production confers resistance to most penicillins, to first, second, and third generation cephalosporins and to monobactams [7, 8, 9]. ESBLs belong to classes A and D of Ambler's beta-lactamases classification and are characterized by containing a serine residue in its active site, in addition to being strongly inhibited by classical inhibitors such as clavulanic acid [10]. ESBLs groups includes nine structural families: TEM, SHV, CTX-M, PER, VEB, GES, TLA, BES and OXA [11]. The TEM, SHV and CTX-M families are the most prevalent worldwide reported in hospital and community infections [6, 12, 13]. ESBLs contained in conjugative plasmids facilitate their mobility through horizontal gene transfer [11, 14, 15] and in turn, these plasmids may contain resistance related genes to other antibiotics, such as quinolones, aminoglycosides, tetracyclines and even colistin, which increases the chances of failure in treatment schemes [16, 17, 18].

ESBL-producing *Enterobacteriaceae* were originally associated with infections related to the hospital environment, however, cases of community-associated infections have increased [5], and ESBL-producing *Enterobacteriaceae* have been reported in sources, such as water, soil, livestock, pets and in healthy human carriers [15, 19]. In the northeast region of Tamaulipas, the presence of ESBL-producing *Enterobacteriaceae* has been reported in animal origin food and superficial waters [20]. However, there is little information regarding the prevalence of such microorganisms of clinical origin. The aim of the present work was to define the molecular epidemiology of ESBL-producing *Enterobacteriaceae* in clinical strains isolated in the city of Reynosa, Tamaulipas in the northeast region of Mexico, in addition to identify the genotype related to ESBL production, determine their microbial resistance profile and lastly, to evaluate their capacity to transfer this resistance.

Results

ESBL-producing Enterobacteriaceae Distribution.

A total of 123 *Enterobacteriaceae* strains were analyzed, of which 61.79% (76/123) presented resistance to CTX, and 48.78% (60/123) were identified as ESBL producers. ESBL-producing strains were mostly found in isolates from diabetic foot wounds (16 strains), urinary tract infections (15 strains), bronchial secretions (8 strains), wounds (7 strains), stool (3 strains), urethral secretions (2 strains), vaginal exudates (2 strains), expectorations (2), bronchial aspiration (2 strains), ulcers (2 strains) and lithotomy (1 strain). Of the identified ESBL producers, *Escherichia coli* (26/60) strains stood out, followed by *Klebsiella pneumoniae* (11/60) among other species as shown in Table 1.

Table 1
Distribution of ESBL-related genes.

Species	Number of strains	Positive strains for some ESBL gene	ESBL Genes					
			bla$_{TEM}$	*bla*$_{SHV}$	bla$_{CTX-M-1}$	*bla*$_{CTX-M-2}$	*bla*$_{CTX-M-9}$	bla$_{CTX-M-8/25}$
Citrobacter koseri	2	2	2	1	2	0	0	1
Edwardsiella tarda	1	1	1	0	0	0	0	0
Enterobacter cloacae	1	1	1	0	1	0	0	0
Erwinia chrysanthemi	2	2	2	0	0	0	0	0
Escherichia coli	26	24	19	0	15	1	2	2
Klebsiella aerogenes	4	4	4	0	3	1	1	3
Klebsiella oxytoca	1	0	0	0	0	0	0	0
Klebsiella pneumoniae	11	10	9	4	6	1	2	3
Providencia stuartii	1	1	1	0	1	0	0	1
Serratia liquefaciens	4	3	2	1	3	0	0	2
Serratia marcescens	2	2	1	0	1	1	1	0
Shigella sonnei	5	5	4	0	3	0	1	3
Total	60	55	46	6	35	4	7	15

ESBL-producing Enterobacteriaceae Genomic Characterization.

Of the 60 phenotypically positive strains, it was possible to identify the presence of genes related to the production of ESBL in 91.67% of the analyzed strains (55/60). The *bla*$_{TEM}$ gene was the most commonly found, in 76. 67% (46/60) of the strains, followed by the *bla*$_{CTX-M-1}$ group with 58.33% (35/60) and the *bla*$_{CTX-M-8/25}$ group with 25% (15/60). Additionally, the *bla*$_{CTX-M-9}$, *bla*$_{SHV}$ and *bla*$_{CTX-M-2}$ genes were found in 11.67% (77/60), 10% (6/60) and 6.67% (4/60) of the strains, respectively. Considering the number of genes per strain, a 40% of the strains (24/60) only presented the presence of one gene, while in 23.33% (14/60) two genes were detected; subsequently, in 15% of the strains (9/60) three genes were identified, in 11.67% (7/60) four genes were detected, while in one strain, five genes related to ESBL production were detected. On the other hand, 3.33% (5/60) of the strains were negative for the surveyed genes.

Table 2
ESBL-producing *Enterobacteriaceae* Resistance Profiles

Species	Number of strains	Resistance Incidence															
		AM	AMC	ATM	CTX	CAZ	CRO	FEP	GM	AN	CIP	NA	LEV	STX	C	FM	TE
Citrobacter koseri	2	2	1	2	2	1	2	2	1	0	1	1	1	2	2	1	1
Edwardsiella tarda	1	1	0	0	1	0	1	1	1	0	1	0	1	1	0	0	1
Enterobacter cloacae	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1
Erwinia chrysanthemi	2	2	2	1	2	0	2	2	1	0	1	2	1	2	1	2	2
Escherichia coli	26	22	15	20	25	21	25	13	10	5	15	13	13	17	10	11	11
Klebsiella aerogenes	4	3	3	4	4	4	2	1	1	0	1	1	1	2	1	1	1
Klebsiella oxytoca	1	1	1	1	1	1	1	1	0	0	1	0	1	0	1	0	0
Klebsiella pneumoniae	11	8	6	11	10	9	8	6	3	1	3	3	3	7	1	6	5
Providencia stuartii	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Serratia liquefaciens	4	4	4	3	4	2	3	1	1	0	2	3	2	3	3	3	1
Serratia marcescens	2	2	2	1	2	2	2	0	0	0	1	2	1	2	2	2	0
Shigella sonnei	5	5	4	5	5	5	4	3	2	1	3	4	3	4	4	5	4
Total	60	51	40	50	58	47	51	31	21	8	30	30	28	41	26	32	28

AM, ampicillin; AMC, amoxicillin with clavulanic acid; ATM, aztreonam; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; GM, gentamicin; AN, amikacin; CIP, ciprofloxacin; NA, nalidixic acid; LEV, levofloxacin; STX, trimethoprim / sulfamethoxazole; C, chloramphenicol; FM, nitrofurantoin; TE, tetracycline.

Antimicrobial Resistance Profiles.

Antimicrobial resistance profiles of the ESBL-producing strains are shown in Table 2. We observed a high resistance to the beta-lactams included in this study, with resistance to CTX being the most common found in 96.67% (58/60) of ESBL-producing Enterobacteriaceae. We can highlight high ranges of resistance to STX, quinolones and TE; while AN turned out to be the most effective antibiotic against the ESBL-producing strains studied in this work. On the other hand, it should be noted that 66.67% (40/60) of the ESBL-producing strains presented the MDR phenotype since it showed resistance to 3 or more classes of antibiotics used in this work.

ESBL Horizontal Gene Transfer.

The conjugation assay was successful (Table 3) in 51.67% (31/60) of the experiments. With CTX-M as the main beta-lactamases type transferred, followed by TEM, while SHV did not succeed in being transferred. It is worth mentioning that 15 strains transferred more than one gene, with 10 (32.26%) and 5 (16.13%) being able to transfer two and three genes, respectively.

Table 3
Distribution and Frequency of ESBL Genes Successfully Transferred by Conjugation

Donor strains	Conjugation successful	Multiple ESBLs transferred	Main ESBL genes transferred					
			bla_{TEM}	bla_{SHV}	$bla_{CTX-M-1}$	$bla_{CTX-M-2}$	$bla_{CTX-M-9}$	$bla_{CTX-M-8/25}$
Citrobacter koseri	2	1	1	0	1	0	0	1
Escherichia coli	15	2	6	0	9	0	0	2
Klebsiella aerogenes	3	3	1	0	3	0	0	3
Klebsiella pneumoniae	5	3	2	0	3	0	1	3
Providencia stuartii	1	1	1	0	1	0	0	1
Serratia liquefaciens	2	2	1	0	2	0	0	2
Shigella sonnei	3	3	1	0	3	0	0	3
Total	31	15	13	0	22	0	1	15

Discussion

ESBL-mediated resistance in *Enterobacteriaceae* is one of the greatest threats to public health at present, increasing morbidity and mortality of many infectious diseases, in addition to increasing health costs. There are different reports in Mexico regarding the presence and mobility of ESBL-producing strains and the genes associated with their production [12, 19, 21, 22, 2, 25]. However, in the northeast region of Mexico only the environmental distribution of these organisms has been elucidated [20].

Our results showed that 48.78% of analyzed *Enterobacteriaceae* presented the characteristic ESBL-production phenotype, being *E. coli* and *K. pneumoniae* the most common species which presented this phenotype; similar results have been reported in different countries [26, 27]. Although *K. pneumoniae* has stood out as one of the most common producers of ESBLs, it should be noted that in recent years, ESBL-producing *E. coli* has gained importance worldwide [28, 29]. Most of the ESBL-producing isolates were isolated from diabetic foot wounds and urinary tract infections (UTIs). As for UTIs, Enterobacteriaceae, specifically *E. coli* stands out as one of the main etiological agents of these sort of infections [30], while in diabetic foot infections *E. coli* and *K. pneumoniae* stands out together with *Pseudomonas aeruginosa*, *Enterococcu*s spp. *Staphylococcus aureus* as common infectious agents related to these wounds. In addition to this, cephalosporins are part of the empirical antimicrobial treatment which could compromise said therapy by prolonging the morbidity of these conditions [30, 31].

Regarding ESBL-related genes, most of the analyzed strains in the present work were found to be related to the TEM type and group 1 of the CTX-M type, while those of the SHV type were found to be less prevalent; although it should be mentioned that the prevalence of ESBL genes varies greatly depending on the geographical region [32]. An interesting fact is that ESBLs of TEM and SHV types had been the main described genetic antimicrobial resistance determinants during much of the 2000s, however, we reported a very low prevalence of the SHV type and an increase in the CTX-M type, which is considered an emerging beta-lactamase. This may be due to the fact that CTX-M type ESBLs come from an environmental *Enterobacteriaceae*, which increases the possibilities of acquiring a CTX-M-producing *Enterobacteriaceae* in the community [28]. An 8.33% (5/60) of analyzed strains presented the ESBL-related phenotype but lacked the searched ESBLs in this work, however, the presence of some different ESBL that was not included in the present work is not discarded.

Lastly, 51. 67% (31/60) of analyzed *Enterobacteriaceae* in this work had the ability to transfer resistance to 3rd. generation cephalosporins, being the CTX-M-1 type beta-lactamase the predominant transferred type in 23/31 trans-conjugating strains, in comparison with the TEM type (13/31). This is relevant due to the CTX-M type beta-lactamase can be mobilized by environmental *Enterobacteriaceae*, and it has been reported that sub-therapeutic concentrations of cephalosporins can increase their ability to transfer genes related to CTX-M beta-lactamases [33, 34]. In addition, 66.67% (40/60) of the ESBL-producing strains showed resistance to more than 3 antibiotic families, which indicates the spread of MDR phenotype in ESBL-producing *Enterobacteriaceae* which could drastically reduce treatment options [4]. Amikacin stood out as the most effective antibiotic, which has already been reported in previous works and this can constitute a treatment option of infections caused by these antimicrobial-resistant microorganisms [35, 36].

To our knowledge, this is the first work that focuses on studying production of ESBLs, genomic type and evaluation of horizontal gene transfer potential of ESBL genes in clinical *Enterobacteriaceae* strains in the northeast region of Tamaulipas. Described findings can give us an insight of the current outlook regarding the state of resistance to 3rd generation cephalosporins and other families of antibiotics in the region. Collected data will allow us to develop possible treatment schemes and to evaluate key points related to the current antimicrobial resistance in the region.

Methods

Strain Selection.

Strains were obtained from the Environment-Microorganism Interaction Laboratory's biobank of the Centro de Biotecnología Genómica of the Instituto Politécnico Nacional and private laboratories of the city of Reynosa, Tamaulipas. A total of 123 *Enterobacteriaceae* strains were selected from bronchial aspiration samples (6), vaginal exudates (5), expectoration samples (5), stool samples (14), wounds (6), lithotomy (1), urine samples (37), diabetic foot wounds (42), bronchial secretions (3), ulcers (3), and urethral secretions (1). Strains were isolated and identified during the period from August 2018 to December 2019.

ESBL Phenotypic Detection Assays.

Strains were subcultured in tryptic soy agar medium (BD Becton Dickinson & Co) supplemented with 2 µg./mL. of cefotaxime (CTX). Those presenting resistance to cefotaxime (CTX) were tested to detect phenotypic ESBL production by double-disk synergy test (DDST) using cefotaxime (CTX 30 µg.), ceftazidime (CAZ 30 µg.), aztreonam (ATM 30 µg.) and cefepime (FEP 30 µg.) (BBL™ Sensi-Disc™). This test was performed by placing a disc containing amoxicillin with clavulanic acid (AMC 30 µg.) in the center of the plate to observe the synergy phenomenon when the inhibition halo increased towards the AMC disc, to consider the strain as positive phenotypic [37].

Generation of Antimicrobial Resistance Genetic Profiles.

Antimicrobial resistance profiles were determined by plate diffusion method for ampicillin (AM 30 µg.), AMC, ceftriaxone (CRO 30 µg.), CTX, CAZ, ATM, FEP, amikacin (AK 30 µg.), gentamicin (GM 10 µg.), ciprofloxacin (CIP 5 µg.), levofloxacin (LXV 5 µg.), nalidixic acid (NA 30 µg.), trimethoprim/sulfamethoxazole (SXM 1. 25/23. 75 µg.), chloramphenicol (C 30 µg.) (BBL™ Sensi-Disc™), nitrofurantoin (FM 300 µg.) and tetracycline (TE 30 µg.); tests were performed according to the Clinical and Laboratory Standards Institute (CLSI, 2020) criteria, using *Escherichia coli* ATCC® 25922™ as negative control.

ESBL-related Genes Characterization.

The presence of the bla_{TEM}, bla_{SHV} and bla_{CTX-M} was detected by multiplex PCR [38], the primers used are shown in the Table 4. PCR was performed with a final volume of 15 µL., comprising 1x buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 10 mM of each primer and 5 U of GoTaq® Flexi DNA Polymerase (Promega, USA). The used amplification program consists of a first step of 94 °C for 1 min, 30 cycles of 94 °C for 40 s, 60 °C for 40 s and 72 °C for 1 min, with one last step of 72 °C for 7 min. PCR amplicons were visualized on a 2% agarose gel electrophoresis performed at 100 V for 1 hour and stained with SYBR Gold™ (Thermo Fisher Scientifc, USA) to be observed on a UV KODAK Gel Logic 100 transilluminator.

Table 4
Specific Primers Used for The Detection of ESBL Groups

Target	Sequence (5'–3')	Amplicon size (bp)
TEM	CATTTCCGTGTCGCCCTTATTC	800
	CGTTCATCCATAGTTGCCTGAC	
SHV	AGCCGCTTGAGCAAATTAAAC	713
	ATCCCGCAGATAAATCACCAC	
CTX group 1	TTAGGAARTGTGCCGCTGYA	688
	CGATATCGTTGGTGGTRCCAT	
CTX group 2	CGTTAACGGCACGATGAC	404
	CGATATCGTTGGTGGTRCCAT	
CTX group 9	TCAAGCCTGCCGATCTGGT	561
	TGATTCTCGCCGCTGAAG	
CTX group 8/25	AACRCRCAGACGCTCTAC	326
	TCGAGCCGGAASGTGTYAT	

Conjugation Potential Tests.

Conjugation tests were performed using the sodium azide-resistant *E. coli* strain J53 as a recipient. Both, the donor strain and the recipient strain were grown separately at 37 ºC overnight in Luria Bertani broth (BD Becton Dickinson & Co.). Conjugation tests were carried out mixing equal volumes in a 1:1 ratio of the donor and the recipient strains for their subsequent incubation at 37 °C for 4 hours. After incubation, they were cultured on tryptic soy agar medium plates supplemented with 2 µg./mL. of CTX and 100 µg./mL. of sodium azide to eliminate donor strains. Positive strains were assayed for the detection of ESBL production-related genes.

Declarations

Author contributions

V.B.G. and E.R.M. contributed to the conception and design of this study; J.O.B., I.H.M., I. R. P., W. C. P. and, E.C.G. collected the samples and performed the experiments, E.C.G. wrote the manuscript. V.B.G., G. R., and E.R.M., reviewed the manuscript.

Competing interests

The authors declare no competing interests.

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