

Molecular Characterization of Multidrug-Resistant Bacteria Isolated from a Boyacá Hospital, Colombia

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Abstract

There is a growing number of beta-lactamase extended spectrum bacteria, which inactivate a wide variety of beta-lactam drugs (ESBLs), including third-generation cephalosporins, penicillins, and monobactams, which are commonly identified in nosocomial infections. Genetic variability and molecular approaches have been widely used for bacterial phylogeny and have been applied in bacterial identification. The bacterial isolates were obtained from June 2014 to December 2014 in a second-level hospital in the state of Boyacá-Colombia. Phenotypic identification was performed using the BioMerieux VITEK-2 system® following the standards of the Institute of Clinical and Laboratory Standards. ESBLs genes, including TEM, SHV, CTXM and AmpC, were analyzed, and 11 strains with drug resistance were analyzed. From the isolates, 100% expressed the blaCTX gene, while 18.2% expressed blaSHV and 27.3% expressed blaTEM. The researchers observed two different amplifications of AmpC genes, one that was 170 bp and the other that was 500 bp. The first had a frequency of 100% in the samples and the second had a frequency of 63.6%. Bioinformatic analysis was performed for each sequence. ESBLs-producing strains are considered a major problem due to multi-drug resistance, their implications in nosocomial outbreaks, and their tendency to spread rapidly throughout the world. Therefore, they are considered important clinical markers; consequently, the knowledge of incidence and origin play an important role in the selection of appropriate treatment.

INTRODUCTION

With the widespread use of broad-spectrum antibacterial agents, the resistance problem continues to worsen. Such disproportionate use generates an increasing number of bacterial strains producing extended-spectrum beta-lactamases (ESBLs) (1). These enzymes inactivate a wide variety of beta-lactam drugs, including third-generation cephalosporins, penicillins, and monobactams (2-4). Molecular characterization of these resistance genes has shown a high rate of mutations in the genes that alter the amino acid configuration around the active site of these enzymes (5, 6). Therefore, approximately 200 different natural ESBLs variants, such as 73 TEM-family ESBLs, 46 SHV types, 37 CTX-M types, 18 OXA types, and 20 other ESBLs have now been identified (7-12). In general, ESBLs-producers are resistant to all penicillin, cephalosporin, and monobactam antibiotics (13). For this reason, these enzymes have spread dangerously in large geographic regions (8). The success of this dissemination is probably due to the horizontal transfer of resistance genes (blaTEM, blaSHV, etc.), often-carried in self-transmissible plasmids or mobile

elements, which able to spread horizontally between and within species (14, 15). For example, plasmid-mediated CTX-M type expanded-spectrum beta-lactamases (ESBLs), which have been extensively reported for the past 10 years, are detected mostly in community-acquired pathogens and are associated mainly with *Escherichia coli* (E.coli) (16, 17). According to Villegas et al (18), there are major differences in the world in the incidence of infections caused by ESBL-producing organisms, especially when comparing industrialized with developing countries. Several reasons may account for this disparity: (i) poorer social and economic conditions; (ii) crowded hospitals, frequently with high patient ÷ nurse ratios; (iii) self-prescription of antibiotics, which are sold over the counter in most of South America; and (iv) deficient hospital hygiene, resulting in high rates of colonization and infection with *Klebsiella* spp. This last factor is very important because *Klebsiella* spp. have a particular ability to acquire plasmids determining ESBLs production (18). In Latin America, there is a high rate of ESBLs, although their distribution is still restricted to some genes and to specific locations (18). In Colombia, as

reported by CIDEIM, values ranging from 8 to 11% in *E. coli* and 20-30% in *Klebsiella pneumoniae* (*K. pneumoniae*) ESBLs phenotypes (19) were described as the first cefotaxime type ESBLs (CTX-M-12) in the country (20).

The Infectious Diseases Society of America (IDSA) grouped some species of bacteria in the term *Eskape*: vancomycin-resistant *Enterococcus faecium* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), extended spectrum beta-lactamase producing *K. pneumoniae* (ESBLs), *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Enterobacter* spp., as these species cause public health problems such as high rates of morbidity and mortality in hospitals (21). These bacteria cause a variety of human diseases, including 30% to 35% of all septicemias, more than 70% of urinary tract infections, and many intestinal infections (22, 23).

Since antibiotic prescription patterns vary in different regions, the prevalent genotype of ESBLs is variable (12). Phenotypic variability among strains belonging to the same species also results in some bacterial isolates presenting characteristics that are atypical for a candidate identification. Therefore, accurate identification of bacterial isolates is an essential task for clinical microbiology laboratories (24, 25). Molecular approaches have been extensively used for bacterial phylogeny and have been applied to bacterial identification, including that of environmental and clinical uncultured microorganisms, unique or unusual isolates, and collections of phenotypically identified isolates (24, 26). The aim of this study was to investigate the presence of ESBLs in clinical isolates of ESBLs-producing gram-negative bacilli. Susceptibility to beta-lactam antimicrobials was examined, along with phenotypic analysis of the isolates based on the presence/absence of inducible AmpC beta-lactamases and the genotypes of four ESBLs (*bla* TEM, *bla*SHV and *bla* CTX M). AmpC and 16S rDNA genes were recovered from clinical samples of a hospital center of Boyacá, Colombia.

MATERIALS AND METHODS

Ethics statement

The observational, descriptive cross-sectional study was conducted in the Molecular Epidemiology Laboratory of Universidad de Boyacá, Colombia. The study was considered as low risk and was approved by the ethics committee at Universidad de Boyacá. The research study was classified as safe in accordance with 008430 of 1993 resolution of Ministry of Health and Social Protection

(Colombia). The study always maintained ethical standards, scientific techniques, and administrative norms for health research from Colombia's Ministry of Social Protection, resolution N° 008430 of 1993. The relevant ethical issues in this study were, that the patient was not directly involved, nor was the patient's name, and the clinical information was limited, since the completion of procedures per patient was not required. Thus, informed consent was not necessary.

Bacterial strains

A total of 458 bacterial isolates were collected from June 2014 to December 2014 in a second-level hospital in the Boyacá state, Colombia. Identification of the isolates was performed by the BioMerieux VITEK-2 system. Resistance phenotypes of the ESBLs and carbapenemases were confirmed following the standards of the Clinical and Laboratory Standards Institute (CLSI). *E. coli* strain ATCC 25922 was used as a sensitive control strain, and *K. pneumoniae* strain ATCC 700603 was used as ESBLs producing positive control strain.

Antibiotics and drug susceptibility tests

The following antibiotics were provided by CLSI recommendations: piperacillin/tazobactam and sulbactam/cefoperazone (Toyama Chemical Co., Ltd., Toyama, Japan); cefazolin, cefuroxime, cefotaxime, and cefepime (Farbwerke HoechstAG, Frankfurt, Germany); cefoxitin, ceftazidime, amikacin, gentamicin and levofloxacin (Sigma Chemical Co., St. Louis, Mo); clavulanate (SmithKline Beecham Pharmaceuticals, Surrey, United Kingdom); imipenem and meropenem (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan).

In the drug susceptibility test, fifteen agents (Sulbactam/Cefoperazone, piperacillin/ tazobactam, cefazolin, cefuroxime, ceftazidime, cefotaxime, cefepime, cefoxitin, imipenem, meropenem, amikacin, gentamicin and levofloxacin) were determined by the broth dilution method according to CLSI recommendations. ESBLs, AmpC and carbapenemase-positive strains should be further conducted by phenotypic testing. Expression of ESBL was detected by a double disk test, using ceftazidime, ceftazidime/ clavulanic acid, cefotaxime and cefotaxime/clavulanate at a distance of 2 cm. The diameter of the zones of inhibition of growth was recorded and interpreted as sensitive, intermediate resistant or resistant based on the CLSI guidelines. The interpretation of the results was considered a positive result, an increased greater or equal to 5 mm in halo of

cephalosporine/clavulanic acid to cephalosporin alone. For the expression of carbapenemases, five antibiotics tests (Aztreonam, cefepime, ceftazidime, imipenem and meropenem) were used.

Genotype detection

The DNA extraction protocol started from a single colony cultivated in MacConkey agar by the exhaustion technique, with the aim of selecting a unique genetic profile. Bacterial strains were inoculated into 10 mL of brain heart infusion broth (BHI) dispensed into Falcon tubes overnight at 37°C in constant 180 RPM agitation. The Wizard® Genomic DNA Purification Kit (Southampton, England) was used for bacterial genomic DNA extraction of all the isolates. DNA concentration was determined using a micro-volume spectrophotometer (MaestroNano®).

Amplification of blaAmpC (AmpC-type beta-lactamase), blaCTX-M1 (CTX-M1 type beta-lactamase), blaTEM (TEM-type beta-lactamase), and blaSHV (SHV-type beta-lactamase) was performed using previously described primers (27). To confirm PCR-based identification results, comparative 16S rDNA sequence analysis was performed. Universal bacterial (27F and 1492R) primers (28), were utilized for PCR amplification of 16S rRNA of gDNA extractions from all positive isolates. Details for the primers and PCR conditions are shown in Table 1.

Table 1

Primers and standardized PCR condition used for amplification of resistance genes and 16S rDNA.

Gene	Primers sequence	Annealing Temp (°C)	Fragment (bp)
blaAmpC	5'-ATCAAACTGGCAGCCG-3'	56.9 ^{a,c} , 60 ^b	170
	5'-GAGCCCGTTTATGCACCCA-3'		550
blaCTX-M1	5'-GACGATGTCAGTGGCTGAGC-3'	58 ^a , 51 ^b , 59.1 ^c	499
	5'-AGCCGCCGACGCTAATACA-3'		
blaTEM	5'-AAACGCTGGTGAAGTA-3'	49 ^{a,b,c}	239
	5'-AGCGATCTGTCTAT-3'		
blaSHV	5'-ATGCGTTATATTCGCTGTG-3'	56 ^{a,b,c}	241
	5'-TGCCTTGTATTTCGGGCCAA-3'		
16S	27F 5' AGAGTTTGATCMTGGCTCAG 3' 1492R 5' TACGGYTACCTTGTACGACT 3'	54 ^{a,b,c}	1465

a: *E. coli*; b: *P. aeruginosa*; c: *K. pneumoniae*

PCR was performed in a 25-mL reaction mixture containing 12.5 mL of 2 X PCR Master Mix (Applied Biological Materials Inc., Richmond, Canada), 0.4 mM of each primer, and 1 µL of sample DNA. The PCR conditions used were initial denaturation at 95°C for 5 min, cyclic denaturation at 95°C for 4 min, annealing at variable temperature (details as Table 1) for 1 min, elongation at 72°C for 1-2 min for 35 cycles and final extension at 72°C for 10 min in a thermocycler (Gradient LTCG-48-101 Labocon; Hampshire, UK). PCR products were detected by a 1% agarose gel.

Positive amplicons were purified by Promega Wizard SV Gel and PCR Clean-up System (Promega Co., Madison, WI) and sequenced by Applied Biological Materials Inc. (Richmond, Canada).

Bioinformatics analysis

DNA sequencing quality was verified using Finch TV ver. 1.4.0 (Geospiza, Seattle, WA, USA). Profiles with overlapping sequences were discarded. DNA sequences were annotated using the BLAST program (<http://blast.ncbi.nlm.nih.gov>) to identify the gene subtypes. Homologous sequences identified with a threshold E-value close to zero and identities > 90% and reference sequences were retrieved from NCBI RefSeq and GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). All sequences were manipulated using the Geneious platform (29). The Multiple sequence alignment was computed by the MUSCLE program (30). Neighbor-joining (NJ) phylogenetic trees were constructed with the Bootstrap method (500 replications) and pairwise deletion with the Molecular Evolutionary Genetics Analysis software (MEGA 6) (31).

RESULTS

Classification and Drug Susceptibility test

Drug susceptibility tests were conducted for 458 bacterial isolates, and 298 of them were negative cultures. Of the remaining 160 positive cultures, 12.5% (20/160) were classified as gram-negative and 87.5% (140/160) as gram-negative bacteria. Bacterial identification shows that, of the total of isolates, 80% (113/140) are *E. coli*, 5% (7/140) are *K. pneumoniae*, 3.6% (5/140) are *Proteus mirabilis* (*P. mirabilis*) and 2.1% (3/140) are *P. aeruginosa*. In a small percentage (9.3%), other bacterial species as *Proteus* sp, *Serratia* sp, *Enterobacter* sp, *Burkholderia* sp, *Morganella* and other *Pseudomonas* sp were identified. This study indicated that the drug resistance rates of the 160 isolates to ampicillin, sulbactam, Cephalothin, Cefuroxime, Cefuroxime Axetil, Cefotaxime and Ceftriaxone were all high (above 90%). In case of other antibiotics, the sensitivity rate of Ceftriaxone and Cefepime (9.1%) was lower than that of Ceftazidime (18.2%).

Detection of ESBLs producing and AmpC producing strains

Among the 160 isolates classified as gram-negative, 11 strains produced both ESBLs and AmpC enzymes. In the 11 strains with the ESBLs phenotype, the diameters of the

zones of inhibition of some or all of the substrates were observed. In addition, the observation of the synergistic effect produced between the broad-spectrum cephalosporins and clavulanic acid; confirms this resistance. Of these resistant strains, 81% (n=9) were isolated from the urine culture, and most were identified as *E. coli* (n=8), while 9% were isolated from the ulcer and wound secretions, respectively, both of which were identified as *P. aeruginosa*.

PCR Amplification of resistance genes and Sequence analyses

To analyze the drug resistance-related genes, PCR amplification and sequencing analysis was conducted for 11 ESBLs isolates. ESBLs genes, including TEM, SHV, CTXM and AmpC type, were amplified from *P.aeruginosa*, *E. coli* and *K. pneumoniae* species that were detected. There were 11 (100%) blaCTX type positive strains, which is related to the producer phenotype of beta-lactamase extended spectrum. These results are summarized in the Table 2. The sequenced analysis of the results by BLASTN showed that blaCTX-M contained two subtypes, including blaCTX-M-15 (n=9) homologous for *E. coli* and *K. pneumoniae* (E value: 0.0; Identity: 100%) and blaCTX-M-2 (n=2) homologous for *P.aeruginosa* (E value: 0.0; Identity: 98%).

Table 2

Molecular characterization of multi resistant isolates and their relationship of origin.

N. Sample	species	Sample origin	Resistance phenotype	bla TEM	bla SHV	bla CTX MI	AmpC	16S	Hospital service
Sample 1	<i>P. aeruginosa</i>	ulcers secretion	ESBLs	-	-	+	170pb 550pb	+	Internal Medicine Service
Sample 2	<i>E. coli</i>	Urine	ESBLs / CBP-KPC	-	-	+	170pb 550pb	+	External consult Service
Sample 3	<i>P. aeruginosa</i>	wound secretion	ESBLs / CARBAPENEMS	-	-	+	170pb 550pb	+	Emergency service
Sample 4	<i>E. coli</i>	Urine	ESBLs	-	-	+	170pb	+	External consult Service
Sample 5	<i>E. coli</i>	Urine	ESBLs	+	+	+	170pb 550pb	+	Emergency service
Sample 6	<i>K. pneumoniae</i>	Urine	ESBLs	-	-	+	170pb	-	Pediatrics emergency
Sample 7	<i>E. coli</i>	Urine	ESBLs	+	+	+	170pb	+	Emergency service
Sample 8	<i>E. coli</i>	Urine	ESBLs	-	-	+	170pb 550pb	+	External consult Service
Sample 9	<i>E. coli</i>	Urine	ESBLs	-	-	+	170pb 550pb	+	Emergency service
Sample 10	<i>E. coli</i>	Urine	ESBLs	-	-	+	170pb	-	Emergency service
Sample 12	<i>E. coli</i>	Urine	ESBLs	+	-	+	170pb 550pb	-	External consult Service
				27.3%	18.2%	100%	100% ^a 63.6% ^b	72.7%	

+; Positive PCR result; -; Negative PCR result; a: AmpC amplification to 170pb; b: AmpC amplification to 170pb

Three (27.3%) blaTEM-1 positive strains that were homologous sequences with *E. coli* (E value: 0.0; Identity: 99%) and two (18.2%) blaSHV positive strains that were homologous sequences with blaSVH-1 subtype of *K. pneumoniae* (E value: 0.0; Identity: 99%) and *E. coli* (E value: 0.0; Identity: 98%) were observed. Similarly, two strains of *E. coli*, which were derived from urine samples (samples 5 and 7) from an external consult service and sent to another emergency department, showed amplification of

blaTEM, blaCTX-M, blaSHV and AmpC genes. In the case of strains identified as *P.aeruginosa* and *K.pneumoniae*, an amplification for AmpC and blaCTX-M genes was presented, but there was no positive amplification for blaTEM and blaSHV genes (Table 2); *P.aeruginosa* has natural resistance type AmpC, which is encoded in the chromosome of the bacteria. Additionally, ESBLs encoded by plasmids, which present two types of b-lactamases, were observed in the amplifications. The researchers observed two different amplifications for the AmpC gene, one to 170pb and another to 500pb. The first has a frequency of 100% in the samples and the second has a frequency of 63.6% (Table 2). Bioinformatics analyses showed that the 170pb amplification is a homologous sequence (E value: 5e-41; Identity: 98%) to triosephosphate isomerase gene of *E. coli* and the 550pb is homologous sequence (E value: 0.0; Identity: 99%) to AmpC of *E. coli* and AmpC of *P.aeruginosa* (E value: 1e-118; Identity: 87%). This analysis confirms that the 550pb band corresponds to the AmpC resistance genotype.

Furthermore, 72.7% (8/11) of the PCR products of the 16S rDNA gene and 27.3% (3/11) mismatch results for *E. coli*, *P. aeruginosa* and *K. pneumoniae* were obtained. PCR amplification of the genomic DNA from the extracted organisms generated an amplicon of the expected size (approx. 1.4 Kb) for all the bacterial isolates. In bioinformatics analysis, probable mixed sequences were not found. A total of 8 samples possessed a 16S rDNA sequence with >96% similarity to that of a genus member. The sequence analyses of the results by BLASTN showed that six are homologous for *E. coli* (E value: 0.0; Identity: 96-97%), one to *P.aeruginosa* (E value: 0.0; Identity: 98%) and one to *Staphylococcus aureus* (*S. aureus*)(E value: 0.0; Identity: 96%).

Phylogenetic analysis

Based on our antibiotic resistance and 16S rDNA gene sequences, different homologous genes were identified; which were downloaded from the GenBank database for phylogenetic analysis (Table 3).

Table 3

Resistance and 16S rDNA genes-based identification of 11 ESBLs bacterial isolations.

Gene	Sequence-based identification	GenBank accession No
blaTEM	<i>E. coli</i>	JN188365
	<i>K. pneumoniae</i>	JN193524
blaSHV	<i>E. coli</i>	FJ668798
	<i>K. pneumoniae</i>	FJ668811
blaCTX M	<i>E. coli</i>	KF891471
	<i>K. pneumoniae</i>	CP008929
	<i>P. aeruginosa</i>	GU929917
AmpC	<i>E. coli</i>	AY899338
	<i>K. pneumoniae</i>	AB933352
	<i>P. aeruginosa</i>	AB198756
16S rDNA	<i>E. coli</i>	DQ360844
	<i>S. aureus</i>	AM980864
	<i>P. aeruginosa</i>	AB680390

The phylogenetic tree sub classified 11 blaCTX genes into three subclasses: 1a (Samples 6, 9, 7, 5, 10, 12, 8, 2 and 4), 1b (Sample 3) and 1c (Sample 1). Subclass 1a of the blaCTX genes have a high nucleotide identity with the reference genes in *E. coli* and *K. pneumoniae*. However, the blaCTX sample 1 gene behaves as an ancestral sequence of 1a, blaSHV, blaTEM and some AmpC genes, and blaCTX sample 3 is the ancestral sequence of blaSHV and blaTEM genes. Similarly, the phylogenetic tree sub classified 7 AmpC genes into three subclasses: 2a (Sample 5), 2b (Samples 1, 3 and 8), and 2c (Samples 9, 2 and 12). Subclass 2a is the ancestral sequence of the 1a group, while 2b is a monophyletic group and is a paraphyletic group of 2a. Finally, the blaTEM and blaSHV genes shape 3 and 4 subclasses, respectively. The phylogenetic analysis of the four resistance genes is shown in Figure 1.

Second, the phylogenetic tree sub classified 8 16S rDNA genes into three subclasses: 5a (Samples 7, 5, 8, 9, 2 and 4), 5b (Sample 3) and 5c (Sample 1). The subclass 5a sequences have high nucleotide identity with the reference genes in *E. coli*, 5b with *P. aeruginosa* and 5c with *S. aureus* (Figure 2).

Figure 1. Phylogenetic tree deduced from three ESBL-producing gram-negative bacilli (blaCTX-M, blaTEM and blaSHV) and AmpC genes from Boyacá's Hospital, Colombia by the neighbor-joining algorithm. Reference sequences were chosen by BLAST algorithm based on the homology of the sequencing results. Branch points supported with bootstrap values $\geq 50\%$ are indicated. The scale below shows the substitutions per site. Subclasses of ESBL (1 to CTMX, 3 to TEM and 4 to SHV genes) and AmpC genes (subclasses 2) are indicate by colored boxes.

Figure 1

Phylogenetic tree deduced from three ESBL-producing gram-negative bacilli (blaCTX-M, blaTEM and blaSHV) and AmpC genes from Boyacá's Hospital, Colombia by the neighbor-joining algorithm. Reference sequences were chosen by BLAST algorithm based on the homology of the sequencing results. Branch points supported with bootstrap values $\geq 50\%$ are indicated. The scale below shows the substitutions per site. Subclasses of ESBL (1 to CTMX, 3 to TEM and 4 to SHV genes) and AmpC genes (subclasses 2) are indicate by colored boxes.

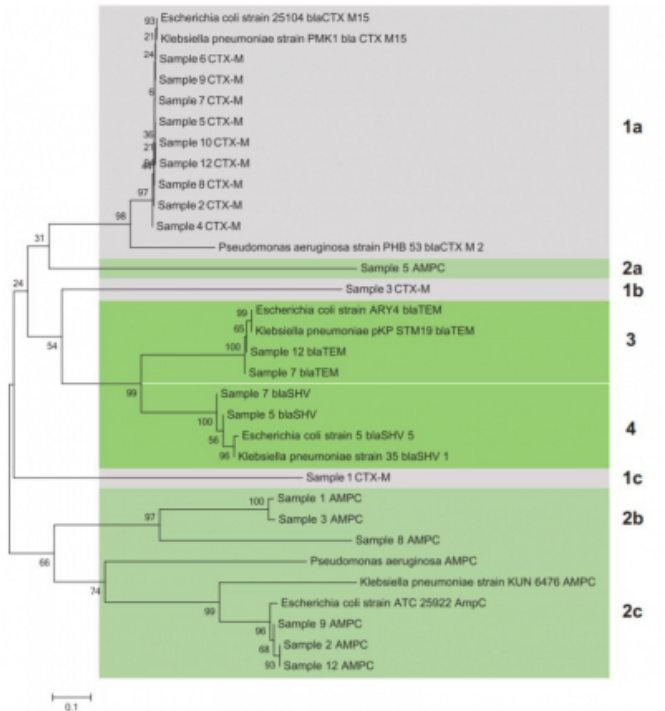
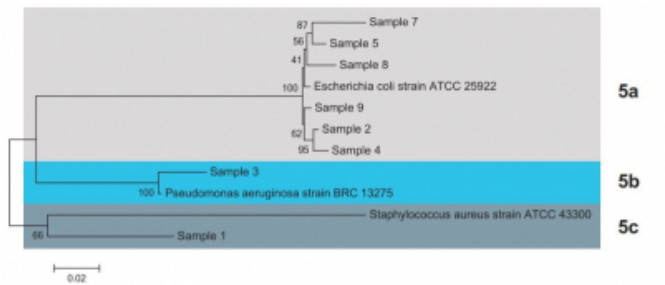


Figure 2

Phylogenetic tree deduced from ESBLs-producing gram-negative bacilli 16S rDNA gene sequences from Boyacá's Hospital, Colombia by the neighbor-joining algorithm. Reference sequences were chosen by the BLAST algorithm based on the homology of the sequencing results. Branch points supported with bootstrap values $\geq 50\%$ are indicated. The scale below shows substitution per site. Subclasses of 16S rDNA gene (5a, 5b and 5c) are indicated by colored boxes.



DISCUSSION

ESBLs producing strains are considered a major problem due to multidrug resistance, its implications in nosocomial outbreaks, and their tendency to spread rapidly around the world. Therefore, they are considered important clinical markers; therefore, knowledge of their incidence and origin plays an important role in selecting the appropriate treatment. Epidemics caused by ESBL-producing bacteria have been reported in most countries, while the prevalence of ESBL-producing strains varies in different countries and hospitals (10, 12). For example, the prevalence of ESBLs producing Enterobacteriaceae was 0% to 25% in the U.S. and 20% to 42% in Europe (CDC National Nosocomial Infections Surveillance, <http://www.cdc.gov/>). In China, the prevalence of ESBL production is high, ranging from 39.2% to 60% (12, 32), 26.4% in Nigeria (33), 40% in Netherlands, 86.6% in India (34), and 40% in Pakistan (35).

Comparatively, those from Sweden (3%) were much less than those from Greece, Turkey, and Portugal (>25%) (36, 37), and a similar comparison could be made for the 1% in Japan (38) compared to South America (>30%) (39-41). A similar study conducted in Colombia on isolates from multiple sources showed higher rates of ESBLs—up to 16.7%—and revealed a few clonally related producers (19). According to some authors, the absence of clonality in most cases suggests that improved infection control will not be an adequate control measure by itself and better antibiotic use will also be required (18).

In this study, a total of 458 bacterial isolates from clinical patients were investigated, of which only 140 were classified as gram-negative. Additionally, the results show that the prevalence of ESBLs producing gram-negative bacilli was 7.8% (11/140), which is lower than those reported in some countries. The proportion of ESBLs positive cases was highest, followed by AmpC-producing stains, and carbapenemases-producing stains. Furthermore, this study indicated that ESBLs positivity was closely related to the resistance of most drugs. The present study suggests that the resistance of ESBLs producing gram-negative bacilli is very serious. ESBLs producing species identified in this study were *E.coli*, *K. pneumoniae* and *P.aeruginosa*, which occurred in 80.7%, 5% and 2.1% of cases, respectively. *P. aeruginosa* has a natural resistance of type AmpC, which is encoded in the chromosome of the bacteria. Additionally, ESBLs encoded by plasmids, which presents two types of beta-lactamases were observed in the amplifications. The degree of resistance depends on the degree of repression of Amp-C. ESBLs are primarily produced by the

Enterobacteriaceae family of gram-negative organisms, in particular *K.pneumoniae* and *E. coli* (27, 37, 42). For example, based on recent multi-continent surveys, *Klebsiella* isolates from Latin America have the highest ESBLs prevalence in the world (45.4–51.9%) (43, 44). The prevalence among *E. coli* isolates, ranging from 8.5% to 18.1% in Latin American countries, was also higher than in developed countries (45). However, over time, the incidence of ESBLs producing bacteria has increased dramatically. In a multicenter study conducted in eleven Latin American countries, Mendes et al.(46) showed ESBLs rates that exceeded 40% in Colombia, with 27% of the ESBLs in *E. coli* and 44% in *K. pneumoniae*. Similarly, another study in Colombia by Martinez et al (43) found an ESBLs rate of 43% for all the isolates studied, with 46% for *K. pneumoniae* and *E. coli* in 20.5%. Furthermore, in this study, the service most frequently corresponds to outpatient where a large number of urine cultures with ESBLs phenotype isolates and isolation were observed in the emergency services and pediatric emergency, in which *E. coli* was most frequently detected in these samples. The highest occurrence of *E.coli* and *K. pneumoniae* producing ESBLs in this study was from urine samples, 9 (81.8%), followed by other infections, and 2 (18.2%) were generated by *P. aeruginosa*. A much higher prevalence rate of ESBLs producers from urinary isolates of gram-negative bacilli have been previously reported in different countries, such as India (58%) (11), Nigeria (37%) (22), Peru (86,4%) (47) and Colombia (50%) (48). It has been shown that urinary tract infections (UTI) are a major cause of sepsis in the hospital setting and the second cause of community healthcare consultation, constituting approximately 40% of urology services (49).

The detection of genetic determinants associated with drug resistance to gram-negative bacilli isolates is essential for appropriate antimicrobial therapy and infection control. ESBLs and AmpCs have been predominant beta-lactamases that mediate gram-negative bacillus resistance to new broad-spectrum beta-lactam antibiotics. ESBLs are mainly mediated by plasmids, while AmpCs are mainly mediated by the chromosome (50). ESBLs are Class A beta-lactamases and may be defined as plasmid-mediated enzymes that hydrolyze oxyimino-cephalosporins, and monobactams but not cephamycins or carbapenems (8) and exist various genotypes of ESBLs. Of these, the most common are the SHV, TEM, and CTX-M types (51).

CTX-M types are the major phenotypes of domestic ESBLs, which have been reported to be prevalent in the world (52),

followed by the SHV type (53). In this study, among the ESBL-producing *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates, the majority of ESBLs genotypes were blaCTX-M, 9 isolates where the subtype of blaCTX-M-15 and 2 strains were blaCTX-M-2. In the present study, only TEM-1 was detected in a total of three isolates and SHV-1 was detected in two isolates. Its rate was extremely low compared to that reported by the previous studies. The enzyme was initially designated CTX-M-1, thus creating the 'cefotaximase family' (CTX-M family), which now comprises more than 60 enzymes (54, 55). The type of ESBLs expressed by this microorganism has changed in recent years. These enzymes are increasingly more prevalent than the classic TEM and SHV-type ESBLs, because these genes have often been substituted by members of the CTX-M family (9, 56). This group of enzymes is prevalent and endemic in South America (18, 57) and, similarly to this work, some other studies in Argentina, Brazil and Bolivia have reported blaCTX-M genes in a great variety of gram-negative species (40, 57). In Colombia, the CTX-M enzymes, especially Group 1, are being reported more frequently (58, 59), while in studies in Brazil, CTX-M-2 predominates in *K. pneumoniae* associated with outbreaks (60). Of increasing importance is the potential effect of the presence of a CTX-M-type ESBLs on the detection of ESBLs by the clinical microbiology laboratory. Those laboratories, which rely on the resistance to ceftazidime as a surrogate marker for ESBLs production, will likely not be aware of organisms producing CTX-M-type ESBLs (27). Additionally, within *E. coli*, CTX-M-15 producing strains are commonly found and frequently harbor multidrug resistance and virulence determinants (61, 62). In accordance with some studies, worldwide dissemination of blaCTX-M-15 is driven by B2 or D *E. coli* clones associated mainly with urinary tract infections or IncFII plasmids containing multiple antimicrobial drug-resistance platforms that contribute to the spread of CTX-M-15 (16). Therefore, our data suggests that the presence of blaCTX-M-15 in ESBL-producing bacteria is independent of the presence of TEM or SHV genes and could be responsible for this resistant phenotype. Nevertheless, it was not possible to conclude if they were responsible for the ESBLs phenotype or not, once non-ESBLs encoding genes were associated with another gene expressing the extended-spectrum phenotype (63).

Detection of AmpC beta-lactamases in *E. coli* and other bacterial species poses a challenge to microbiological laboratories. Furthermore, plasmid mediated AmpC beta-lactamases represent a new threat, since these confer

resistance to cephamycins and are not affected by beta-lactamase inhibitors, and can provide resistance to carbapenems in strains that have lost outer membrane porins. This resistance mechanism in *E. coli* and *K. pneumoniae* has been found around the world to cause nosocomial outbreaks (64, 65). In the present study, AmpC genes were detected in seven isolates, with a high prevalence (63.6%). In previous research, a high prevalence (35%) in *E. coli* and other bacterial isolates of the AmpC phenotype in Switzerland (66), India (77.5%) (67) and Colombia (48.6%) have been detected (58). Additionally, we detected a PCR product of 170 bp, which was discarded by sequencing the AmpC gene. This 170 bp product, which was previously reported as a positive product of AmpC (58), is actually the triosephosphate isomerase gene; therefore, the prevalence presented by this work is higher than normal. Organisms producing plasmid-mediated AmpC beta-lactamases (PMABLs) such as *E. coli* and *Klebsiella* sp, are often associated with multidrug resistance, leaving few therapeutic options. In addition, the co-existence of ESBLs may mask their detection phenotypically (67). There are no CLSI guidelines available for its optimal detection and confirmation. Therefore, phenotypic tests do not differentiate between chromosomal AmpC genes and AmpC genes that are carried on plasmids. Hence, genotypic characterization is considered the gold standard (68).

Molecular phylogeny increasingly supports the understanding of organismal relationships and provides the basis for the classification of microorganisms according to their natural affiliations. In most genes, our DNA sequence phylogeny confirms the genus and species generated by the phenotypic characterization of the strains studied. However, the phylogeny observed in this study shows that samples 5 and 8 of AmpC, and sample 3 for the CTX-M genes could be an ancestral sequence from *E. coli* and *P. aeruginosa*; presenting a high rate of nucleotide substitution in DNA sequence, without the known subtype. A possible mechanism that could explain this fact is gene conversion in bacteria. One of the strongest pieces of evidence for this mechanism is the study of the pilS gene, which encodes the pilus on *Neisseria gonorrhoeae* (*N. gonorrhoeae*) (69). However, the CTX-M-type ESBLs gene may be transferred by genetic mobile elements such as plasmids, transposons or integrons (12), which could also explain this phenomenon. Similarly, the phylogeny of the 16S rDNA gene confirms most of the previously characterized species. Nevertheless, sample 1 was phenotypically characterized as *P. aeruginosa* but phylogenetically would come from *Staphylococcus*

aureus. This result could be explained by the mechanism of horizontal transfer between different species of bacteria by similar processes to those discussed above in the CTX-M gene. The transfers of DNA by transduction (via bacteriophages) or by transformation (when DNA is released from a bacterium and taken up by another) are not believed to be relevant mechanisms of antibiotic resistance transfer (70). By contrast, conjugation, i.e., direct cell-to-cell contact, can potentially achieve horizontal gene transfer, as it has been shown to be a mechanism with a broad host range (14). For example, conjugation between two multiple antibiotic resistant isolates, *P. aeruginosa* and *E. coli*, as the donors and *E. coli* Rif(r) (sensitive to antibiotics) as the recipient has been demonstrated through experiments (71). However, in our case of the 16S rDNA phylogeny, the horizontal transfer of genes between gram-negative and gram-positive bacteria must be contemplated. This transfer is not uncommon, as natural horizontal gene transfer from gram-positive to gram-negative bacteria has been previously reported (15). Such an event could explain the data observed in the phylogeny of the resistance genes and the 16S rDNA for sample 1. Therefore, comparative sequence analysis of ribosomal RNAs or corresponding genes currently are the most widely used approach for the reconstruction of microbial phylogeny (72, 73), more so in regard to bacteria that have a great impact on public health.

This study has revealed that ESBLs positive isolates from a Hospital of Boyacá, Colombia, were resistant to the majority of new broad-spectrum beta-lactam antibiotics, and some strains also carry the AmpC gene, which together generated a multidrug-resistant strain. Because our study reports a high prevalence of AmpC genes in nosocomial infections, dissemination of these organisms within the hospital or between different regions of the country may become an important public health issue. The molecular characterization of resistance genes and confirmation by 16S has become a fundamental tool in the study and treatment of suspected nosocomial infections with ESBLs producing bacteria worldwide.

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Compliance with ethical standards

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Ethical approval: All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The local ethics committee approved this study (reference number CB165, august 6Th 2015).

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