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ORIGINAL ARTICLE

Characterization of antibiotic-resistant *Escherichia coli* associated with urinary tract infections in Southern Colombia

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Abstract

Bacterial resistance to antibiotics is one of the largest medical concerns worldwide. One of the bacteria of critical priority is *E. coli*, since it presents different resistance mechanisms and some of its strains have evolved resistance to beta-lactam antibiotics. We characterized 32 antibiotic resistant bacterial isolates from confirmed cases of urinary tract infections from an array of patients in Nariño, southern Colombia. Macro and microscopic descriptions of the 32 clinical isolates were conducted. Resistance profiles, biochemical, and molecular characterization (via 16S rRNA gene sequencing, *ERIC-PCR*, and resistance genes) were performed. All the isolates were identified as *E. coli* and had resistance to beta-lactams, aminoglycosides, and fluoroquinolones. This resistance was related to plasmids carrying the blaTEM, blaSHV1, and blaCTXM1 genes. There were significant differences between the resistance proportions of the samples (p value: 0.0000), mainly to penicillin, cefotoxin, and imipenem. Using ERIC-PCR, four clonal states were evidenced that corroborate a degree of genetic differentiation within the isolate set. Antibiotic resistance observed in the isolates is associated with resistance genes present in the bacterial chromosome and plasmids.

Keywords: antibiotics; *Escherichia coli*; urinary infections; plasmids and resistance.

Introduction

Antibiotics are synthetic or semisynthetic compounds that exert specific actions on microorganisms by inhibiting or eliminating their growth. However, a large majority of bacteria have developed mechanisms to counteract the effects of antibiotics, adapting to new pressures in the environment [1]. Resistance to antibiotics is considered a natural

manifestation of bacterial evolution. Inappropriate application and abuse of antibiotics has triggered the rapid evolution of antibiotic resistance in bacteria [2]. In hospital environments, the continuous use of various antibiotics favors the onset of different molecular mechanisms of resistance and the selection of multidrug-resistant bacterial strains [1].

In this regard, the World Health Organization [3] notes that the bacterial species with the greatest concern in hospital settings are *Klebsiella pneumoniae, Enterococcus faecalis, Streptococcus pneumoniae, Shigella* spp., *Salmonella Typhi*, *Mycobacterium tuberculosis*, and *Escherichia coli*, possessing highly efficient mechanisms to acquire, store and share genes associated with antibiotic resistance [3]. On the other hand, resistant *E. coli* is a common cause of urinary tract infections (UTI) in the community and in hospital environments. UTI treatment with antibiotics is established empirically, taking into account the data of local epidemiology and the associated resistance maps [4].

In Colombia, Alviz-Amador and collaborators [5] reported that the bacteria most frequently isolated from patients with UTI in hospitals of Cartagena (Colombia) are *E. coli*, *Pseudomonas aeruginosa*, and *K. pneumoniae*. *E. coli* was the cause of about half (46.7 %) of the studied urine cultures, harboring genes associated with the production of extended-spectrum beta-lactamases (ESBL).

In another hospital setting study, in the department of Nariño (Southern Colombia) [6], 41.7 % of *E. coli* strains revealed prevalent resistance to broad spectrum antibiotics and caused an increase in the frequency of difficult-to-treat infectious diseases. There is, however, little knowledge about the resistance determinants against the main antibiotics used in the medical field.

Although preliminary tests have been conducted to detect antibiotic resistance, a specific study of resistance in relevant bacteria in this environment has not been carried out. Therefore, the present research aims to characterize isolates of *E. coli* resistant to antibiotics associated with urinary tract infections.

Material and methods

Acquisition of isolates

A total of 32 bacterial isolates form an equal number of confirmed UTI cases were obtained from the Limited Specialized Clinical Laboratory of the city of San Juan de Pasto (Nariño, Colombia). Twenty-two of these isolates

had information about sample origin, date of isolation, resistance profile, and, in some cases patient age, ranging from 9 to 70 years of age. Further information on samples and patients was withheld by the laboratory on confidentiality grounds.

Microbiological characterization

The macroscopic description of the 32 bacterial isolates was carried out. For this purpose, the obtained bacterial isolates were initially propagated in Eosin Methylene Blue (EMB) medium. Subsequently, the isolates were grown on Mueller Hinton agar (MH). After 18 hours of incubation at 37 °C, the following culture attributes were described: shape, margin, edge, elevation, surface, texture, optical property, size, configuration, and color. The description of microscopic characteristics was made by Gram staining and the preliminary identification of the isolates was performed using the following specific tests for gram-negative bacteria (*E. coli*): triple sugar iron agar (TSI), sulfide indole motility (SIM), indole, Simmons' citrate (CS), oxidase, lysine iron agar (LIA), and Metil Red-Voges-Proskauer (MR-VP).

Antibiotic resistance profiles of the *E. coli* isolates were obtained, followed by the Kirby-Bauer method (agar diffusion method), using beta-lactam antibiotics (ampicillin-sulbactam, amoxicillin - clavulanic acid, penicillin, cefotaxime, and aztreonam), carbapenems (imipenem) aminoglycosides (amikacin and gentamicin), fluoroquinolones (ciprofloxacin), and trimethoprim/sulfamethoxazole, according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) [7]. Once the resistance of the *E. coli* isolates was confirmed, the construction of a bank of primary cells was carried out, and initial viability was determined in zero time; subsequently, the isolates were conserved at -20 °C.

Molecular characterization

Extraction of chromosomal DNA

The protocol used for the extraction of chromosomal DNA from *E. coli* was proposed by Burbano-Rosero *et al*. [8]. DNA resuspension was performed in 50 μL of milli-Q water. To verify DNA integrity, 1 % agarose gels were run with the molecular size markers *Lambda, HindIII*, and 1-kb (Promega, USA). The Ez Vision product (AMRESCO-Solon, USA) was used as an intercalator. Run conditions were 70 V for 1 hour and 30 minutes in a Multisub Electrophoresis System Cleaver Scientific chamber. The gel was visualized in a SmartDoc Imaging Enclosure Benchmark Accuris E300 UV photodocumentation system at a wavelength of 302 nm. The DNA concentration was measured with a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer.

Extraction of plasmid DNA

The extraction of plasmid DNA was carried out with the commercial kit PureYield™ Plasmid Miniprep System (Promega, USA) following the manufacturer's instructions. To verify the integrity of the extracted plasmid DNA, 1.2 % agarose gels electrophoresis was run. A 1-kb molecular ladder (Promega, USA) was employed as molecular size marker and the gel was stained with the DNA intercalator Gel Red (Biotium, USA). The run tool place at 80 V for 1 hour and 30 minutes in a Multisub Electrophoresis System Cleaver Scientific chamber. The gel was visualized in a SmartDoc Imaging Enclosure Benchmark Accuris E300 UV photo documentation system at a wavelength of 302 nm. Consecutively, a dendrogram was built, with the number of plasmid bands and their sizes, using the NTSYS Spc 2.1 software (License UH3071IX) and following the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method.

Amplification of genes from chromosomal and plasmid DNA linked to antibiotic resistance

To assess the presence of antibiotic resistance genes in bacteria from UTI isolates, PCR amplification of a set of chromosomal and plasmid resistance genes was performed. Primer sequences were obtained from the literature (**Table 1**). PCR conditions, per target gene, are shown in **table 2**.

Amplification of the 16S RNAr

The primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') [9] and 1041R (5' CGG TGT GTA CAA GAC CC 3') [10] were used to PCR amplify the 16S RNAr gene. In each reaction, these primers were employed at a final concentration of 0.4 mol/m^6 in addition to 0.05 mol/m^3 dNTP mix, 0.025 $U/\mu L$ Taq DNA Polymerase, 21 mol/m^3 $MgCl_2$, 10 μL of Colorless Buffer (Promega, USA), and 32.75 μL of ultrapure H_2O. PCR run was performed on a MULTIGENE Labnet thermocycler, with the following thermocycling program: 95 °C for 2 minutes, 30 cycles of 94 °C for 2 minutes, 55 °C for 1 minute and 72 °C for 3 minutes; and a final extension of 10 minutes at 72 °C. Amplicons were revealed by 1 % agarose gel electrophoresis; employing a 1 kb DNA Ladder (Promega, USA) as molecular size marker, and Gel Red (Biotium, USA), as an intercalator. The run conditions were 70 V for 1 hour and 30 minutes in Multisub Electrophoresis System Cleaver Scientific chamber. The gel was visualized in a SmartDoc Imaging Enclosure Benchmark Accuris E300 UV photodocumentation system at a wavelength of 302 nm [8].

Table 1. Primers for resistance gene amplification.

Genes	Primers	Authors
blaCTX-M	CTXM-1 (5'-TTTGCGATGTGCAGTACCAGTAA 3')	[9]
	CTX-M-2 (5' CGATATCGTTGGTGGTGCCAT 3')	
blaKPC **(Carbapenemases)**	KPC- F (5' CGTCTAGTTCTGCTGTCTTG 3')	[10]
	KPC-R (5' CTTGTCATCCTTGTTAGGCG 3')	
blaCTX-M-2 **(Cefotaxime)**	CTX-M-2F (5' ATG ATG ACTCAG AGC ATT CG 3')	[9]
	CTX-M-2R (5' TTA TTG CAT CAG AAA CCG TG 3')	
blaTEM **(BLEE)**	TEM -1 (5' ATAAAATTCTTGAAGACGAAA 3')	[11]
	TEM- 2(5' GACAGTTACCAATGCTTAATCA 3')	
blaSHV **(BLEE)**	SHV --1(5' TCAGCGAAAAACACCTTG 3')	[11]
	SHV - 2(5' TCCCGCAGATAAATCACCA 3')	
blaSHV **(BLEE)**	S H V - 1 (5' TGGTTATGCGTTATATTCGCC 3')	[11]
	S H V - 2 (5' GGTTAGCGTTGCCAGTGC 3')	
blaKPC **(Carbapenemases)**	KPC-f-(5' CC GTCTAGTTCTGCTGTC 3')	[12]
	KPC-r (5' CGTTGTCA TCC TTGTTAG 3')	
Mcr-1 **(Colistin)**	mcr-1-F (5' ATGATGCAGCATACTTCTGTG 3')	[13]
	mcr-1-R (5' TCAGCGGATGAATGCGGTG3')	
FosA **(Fosfomycin)**	fosA–F131(5' ATCTGTGGGTCTGCCTGTCGT 3')	[14]
	fosA–R401 (5' ATGCCCGCATAGGGCTTCT 3')	

Sequencing of 16S RNAr

16S RNAr amplicons were prepared and sent to Corpogen (Bogota, Colombia) following the provider's instructions (CONTRACT: 190301-1). Sanger sequencing of the samples was performed in an ABI PRISM®3500 Analyzer sequencer (8 capillary type).

Table 2. PCR conditions for resistance gene amplification.

Genes	Conditions
blaCTX-M	94 °C 2 min 30 cycles of 94 °C 30 s, 56 °C 1 min, 72 °C 1 min and final extension to 72 °C for 5 min.
blaKPC (Carbapenemases)	94 °C 2 min 30 cycles of 94 °C 30 s, 54 °C 1 min, 72 °C 1 min and final extension to 72 °C for 5 min.
blaCTX-M-2 (Cefotaxime)	94 °C 2 min 30 cycles of 94 °C 30 s, 54 °C 1 min, 72 °C 1 min and final extension to 72 °C for 5 min.
blaTEM (BLEE)	94 °C 2 min 30 cycles of 94 °C 30 s, 50 °C 1 min, 72 °C 1 min and final extension to 72 °C for 5 min.
blaSHV (BLEE)	94 °C 2 min 30 cycles of 94 °C 30 s, 52 °C 1 min, 72 °C 1 min and final extension to 72 °C for 5 min.
blaSHV (BLEE)	94 °C 2 min 30 cycles de 94 °C 30 s, 52 °C 1 min, 72 °C 1 min and final extension to 72 °C for 5 min.
blaKPC (Carbapenemases)	94 °C 2 min 30 cycles of 94 °C 30 s, 54 °C 1 min, 72 °C 1 min and final extension to 72 °C for 5 min.
Mcr-1 (Colistin)	94 °C 2 min 30 cycles of 94 °C 30 s, 52 °C 1 min, 72 °C 1 min and final extension to 72 °C for 5 min.
FosA (Fosfomycin)	94 °C 2 min 30 cycles de 94 °C 30 s, 56 °C 1 min, 72 °C 1 min and final extension to 72 °C for 5 min.

Editing sequences

Retrieved forward trace files of the 16S RNAr genes were visualized in the program Chromas lite V. 2.01; the sequence files were edited and aligned using the program BioEdit V. 7.0.4 [8].

Comparison with the Ribosomal Database Project (RDP) and GenBank database NCBI

Initially, the comparison of the sequences was carried out in the Ribosomal Database Project (RDP) and National Center for Biotechnology Information

(NCBI) (www.ncbi.nlm.nih.gov/genbank/). Once the results were obtained, the accession exhibiting the highest percentage of identity and origin to the analyzed sequences was selected.

Enterobacterial repetitive intergenic consensus-PCR

To further asses the identity of the bacterial isolates from UTI samples, the molecular fingerprint of the enterobacterial repetitive intergenic consensus (ERIC) region was studied via PCR. The PCR primers employed were ERIC 1 (5' TGTAAGCTCCTGGGGAT3') and ERIC 2 (5'AAGTAAGTGACTGGGGGTGAGC 3 ') [11]. Each reaction was conducted at a final volume of 50 μL and consisted of 0.4 mol/m^6 of primers, 0.05 mol/m^3 dNTP mix, 0.025 U/μL Taq DNA Polymerase, 21 mol/m^3 $MgCl_2$, 10 μL of Colorless Buffer (Promega, USA), and 32.75 μL of ultrapure H_2O. The samples were run in a MULTIGENE Labnet thermocycler with the following program: 95 °C for 7 minutes; 30 cycles of 94 °C for 1 minute, 52 °C for 1 minute, 65 °C for 8 minutes; and a final extension at 65 °C for 15 minutes [11]. The amplicons were inspected in electrophoresis in 1 % agarose gels– (1X TAE); the 1 kb DNA ladder (Promega) was used as a molecular marker, and Gel Red (Biotium, USA) was used as an intercalator. The run conditions were 70 V for 90 minutes in a Multisub Electrophoresis System Cleaver Scientific chamber. The gel was visualized in a SmartDoc Imaging Enclosure Benchmark Accuris E300 UV photodocumentation system at a wavelength of 302 nm. Subsequently, the polymorphisms were analyzed with the NTSYS Spc 2.1 software (License UH3071IX).

Statistical analysis

A descriptive analysis of *E. coli* resistance to different antibiotics was performed using tables and frequency histograms. In addition, a chi-square test was performed to evaluate the hypothesis that the mean proportions of the samples for the ten antibiotics are completely identical. Likewise, we investigated whether means were significantly different via analysis of means (ANOM).

Results and discussion

Microbiological characterization

The macroscopic and microscopic characteristics of the 32 *E. coli* isolates were determined. According to macroscopic morphological observations, the isolates formed white, small, and circular colonies showing entire margins with elevated, smooth, creamy, bright, and round configurations. The microscopic observations revealed the presence of gram-negative bacilli.

Most bacterial isolates exhibited similar biochemical characteristics, being positive for the production of indole, lactic, formic, and succinic acids, as well as, production of gas, glucose fermentation, lactose, and sucrose. Mobility was another feature observed in the bacteria. The isolates exhibited a negative response for the production of di-acetyl, cytochrome oxidase, and hydrogen sulfide. The bacteria were unable to use citrate as a sole carbon source and fail to carry out lysine decarboxylation.

Isolates 3, 7, 8, 10, and 15 revealed variation in the indole and gas production tests, possibly because these bacteria possess mechanisms for modifying gene expression depending on the needs and their environment. The activation or repression of certain genes is generally reflected in differences at the phenotypic level [12]. Considering all these microbiological characterization data and following the Bergey's Manual of Systematic Bacteriology [13] we established that all of the isolates belonged to the species *E. coli.*

Of the 32 bacterial isolates, 71.87 % were resistant to more than three antibiotics and 62.5 % were resistant to 3 or more antibiotic groups (**Fig. 1** and **Fig. 2**). The group of isolates had the following degrees of antibiotic resistance: penicillin (100 %), trimethoprim-sulfamethoxazole (65.62 %), ciprofloxacin (62.5 %), amoxicillin - clavulanic acid (56.25 %), ampicillin-sulbactam (50 %), gentamicin (43.17 %), aztreonam (28.13 %), amikacin (25 %), cefotaxime (18.75 %) and imipenem (0 %).

The obtained antibiotic resistance profiles can be compared with those observed by Yábar *et al*. [14], where strains of *E. coli* isolated from hospital environments in Lima, Peru, showed marked resistance to antibiotics such as trimethoprim-sulfamethoxazole, ampicillin-sulbactam, and ciprofloxacin and sensitivity to amikacin and carbapenems. Through statistical analysis, we identified highly significant differences between the resistance proportions of the samples (p-value: 0.0000). We also identified that the biggest differences were associated with penicillin, cefotaxime, and imipenem (**Fig. 3**).

In addition, Luna-Pineda *et al*. [15] noted that strains of *E. coli* isolated from children with UTI exhibited multiresistance patterns. Alviz-Amador *et al*. [5] indicated that at the University Hospital of the Caribbean in Cartagena, one of the main pathogens causing urinary tract diseases was *E. coli* and presented a profile of susceptibility to ampicillin-sulbactam (57.8 %), trimethoprim sulfamethoxazole (51.7 %) and ciprofloxacin (34.2 %). In our case, two of the isolates studied, came from underage patients exhibiting resistance to penicillin and amoxicillin. Additionally, one these two cases, a 16-year-old, had resistance to ampicillin-sulbactam and the other, a 9-year old patient, was resistant to trimetropim sulfamethoxazole and gentamicin.

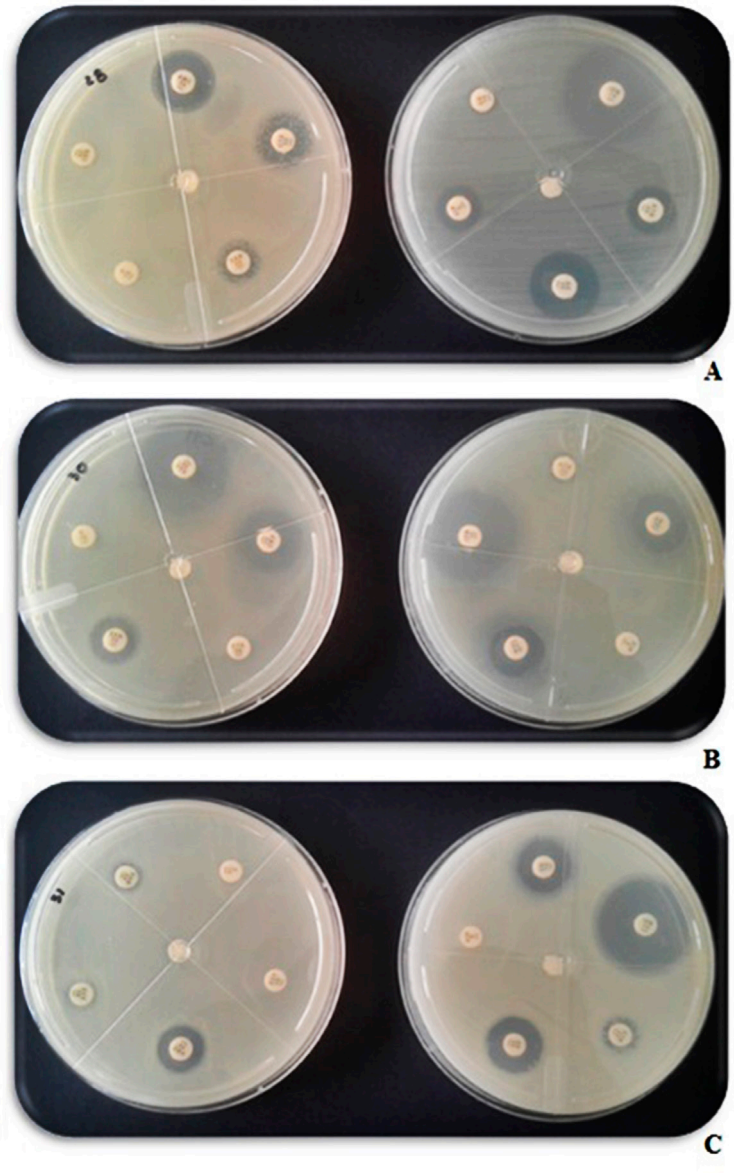

Figure 1. Antibiotic resistance profiles (beta-lactams, carbapenems, aminoglycosides, fluoroquinolones, phosphonates, and trimethoprim/sulfamethoxazole) of three *E. coli* isolates; **A**. isolate 28, **B**. isolates 30, and **C**. isolate 31.

Nocua-Báez *et al*. [16] determined that in nine hospitals in Colombia, all samples of *E. coli* isolated from the urinary tract of diabetic patients and pregnant women (75 % of patients were over age 50) showed resistance to amikacin and imipenem. In the present study, seven individuals, ages 32 to 57, exhibited different degrees of antibiotic resistance: penicillin (100 %), ampicillin-sulbactam (85.71 %), amoxicillin (42.2. 85 %), trimetropim sulfametoxazole (57.14 %), gentamicin (42.85 %), amikacin (57.14 %), and aztreonam (28.57 %). On the other hand, Mahalingam *et al*. [17] evaluated

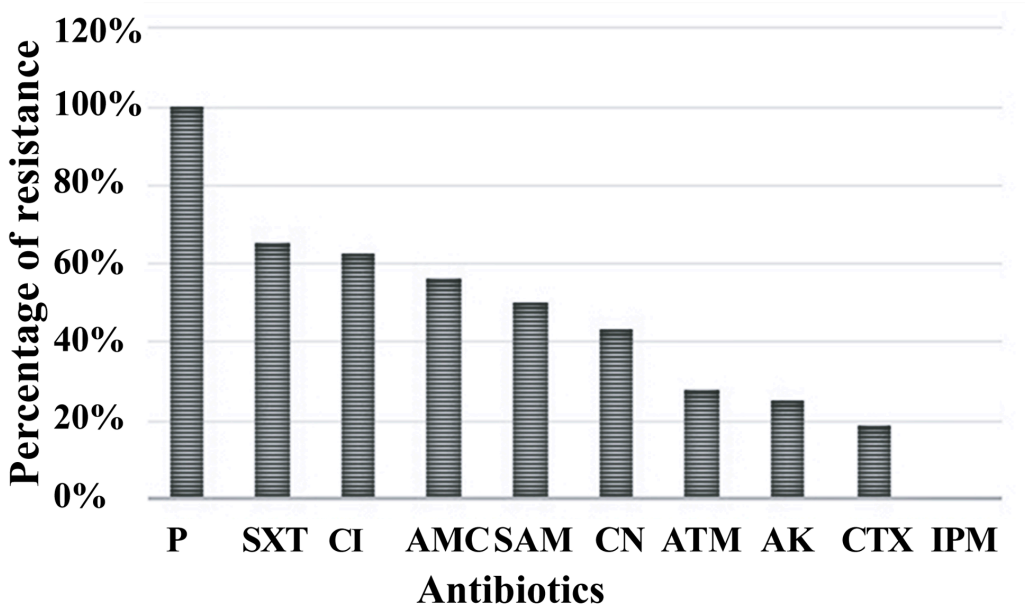

Figure 2. Resistance profile of *E. coli*. isolates against P: penicillin; SAM: ampicillin-sulbactam; AMC: amoxicillin - clavulanic acid; CTX: cefotaxime; SXT: trimethoprim-sulfamethoxazole; CN: gentamicin; ATM: aztreonam; CIP: ciprofloxacin; IPM: imipenem; and AK: amikacin.

profiles and resistance genes at a hospital in South India of *E. coli* samples from patients between an age range of 50 to 70 years, concluding that the isolates were chiefly resistant to cephalosporins and aminoglycosides; likewise, Luna-Pineda *et al*. [15] noted that strains of *E. coli* isolated from children with UTI exhibited multiresistance patterns. Similarly, we found that three individuals of age 60 to 70 showed profiles of resistance to penicillin (100 %), ciprofloxacin and trimetropim sulfametoxazole (66 %), amoxicillin, aztreonam, and cefotoxin (33 %). Furthermore, in our study, antibiotic resistance profiles determined in individuals aged 71 to 90 years were as follows: penicillin (100 %), ciprofloxacin (80 %), ampicillin-sulbactam, amoxicillin and trimetropim sulfametoxazole (70 %), gentamicin (50 %), aztreonam (40 %), cefotoxin (30 %) and amikacin (20 %).

Taking into account the studies described above including ours, in Colombia, reveal common features. However, resistance profiles appear to be unique to each of the areas evaluated, if the environment in which these microorganisms develop is taking into account. According to Bryce *et al*. [18], two of

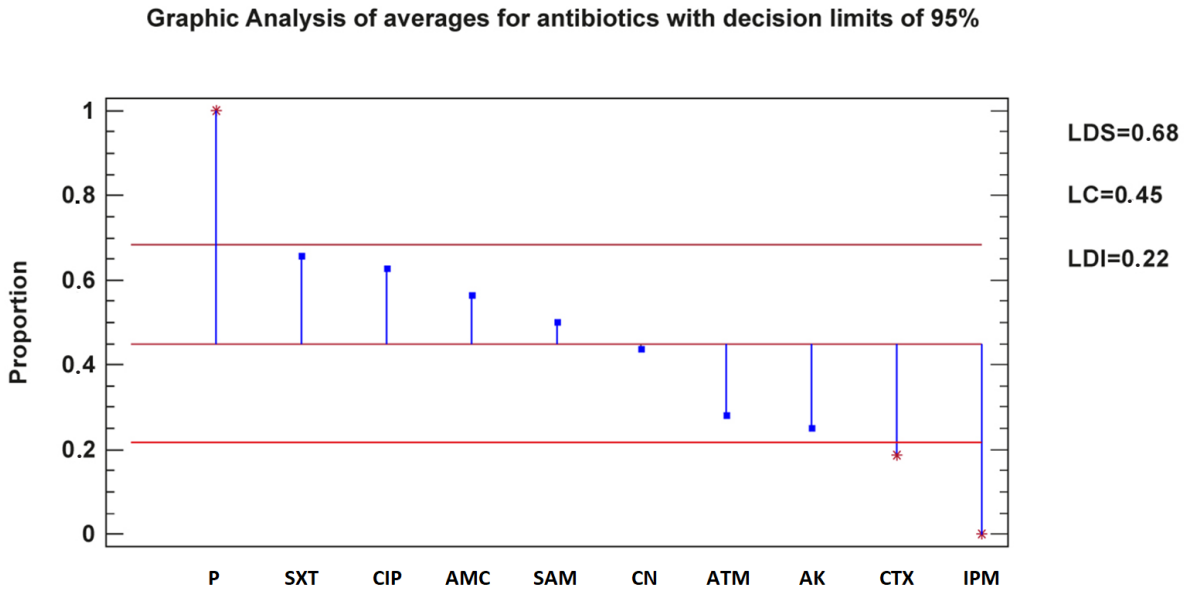

Figure 3. Statistical analysis, using the chi-square test, of the proportions of *E. coli* isolates resistant to the following array of antibiotics. P: penicillin; SAM: ampicillin-sulbactam; AMC: amoxicillin - clavulanic acid; CTX: cefotaxime; SXT: trimethoprim-sulfamethoxazole; CN: gentamicin; ATM: aztreonam; CIP: ciprofloxacin; IPM: imipenem; and AK: amikacin.

the main factors leading to geographical variation in antibiotic resistance profiles are prolonged exposure to antibiotics and use of antibiotics without medical prescription in some part of the globe. Rada *et al.* [19] noted that Colombia does not differ significantly from global antibiotic resistance trends and is becoming an endemic country of carbapenemase enzymes thanks to a high degree of exchange elements such as plasmids. In this context, we gather that the high percentages of resistance to beta-lactam antibiotics and quinolones arise because these are first-line treatments to counteract urinary tract infections caused by *E. coli*. Third generation cephalosporins and carbapenems presented low percentages of resistance, so they can be considered as optional treatments for these diseases.

Extraction of chromosomal DNA

All of the DNA samples showed the adequate quality (absorbance ratio 260/280 between 1.7 and 2.0) and concentrations (greater than or equal to 100 ng/μL) (**Suppl. 1**). The samples were adjusted to a concentration of 50 ng/μL.

Extraction of plasmid DNA

Of the 32 *E. coli*, isolates, 16 had plasmids with one to five bands per sample and approximate sizes between 0.91 and 18.7 kilobases; the most common plasmid sizes among samples were 10.7 and 1.05 kb; only two samples exhibited plasmids larger than 16 kb (**Suppl. 2** and **Suppl. 3**). The analysis of similarity with respect to the presence of plasmids indicated that most of the isolates have a similarity greater than or equal to 50 %. Isolates 26 and 20 exhibited a similarity percentage of 80 % (**Fig. 4**) since they share two plasmids with sizes of 10 706 and 1,05 kilobases, respectively. This agrees with the resistance of the two isolates to antibiotics such as amoxicillin - clavulanic acid, penicillin, and trimethoprim-sulfamethoxazole.

Based on the dendrogram obtained, four subclusters with percentages of similarity greater than 50 % were distinguished. This is directly related to the presence of unique and common plasmids in each of the groups.

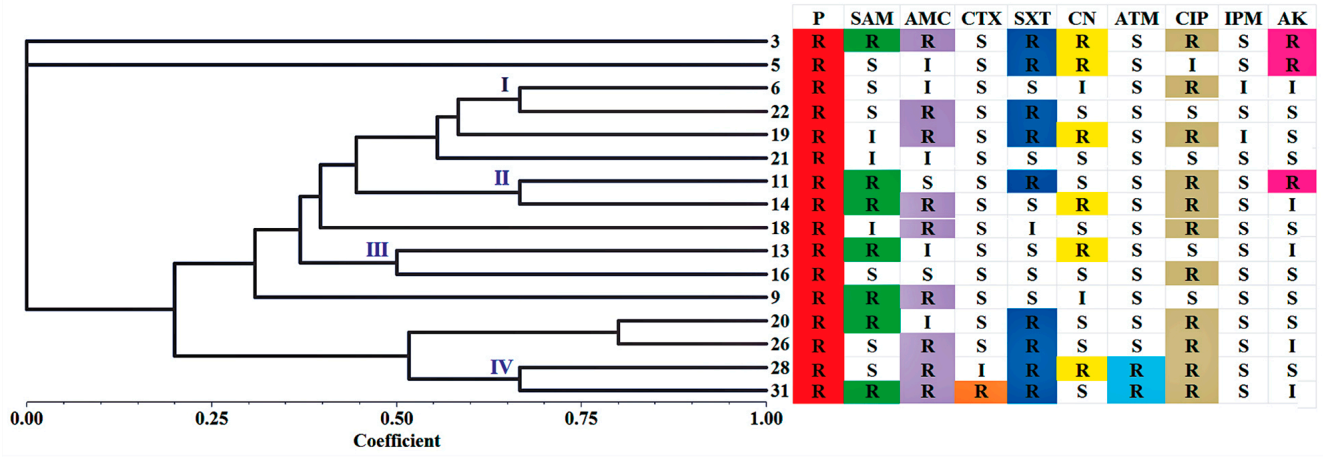

Figure 4. Dendrogram of the plasmid profile of *E . coli* isolates. The dendrogram was built using the NTSYS Spc 2.1 software following the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. Antibiotic resistance is color-coded. Red (resistance to Penicillin), green (resistance to amoxicillin), orange (resistance to cefotaxime), blue (resistance to trimethoprim-sulfamethoxazole), yellow (resistance to gentamicin), light blue (resistance to aztreonam), light brown (resistance to ciprofloxacin) and fuchsia (resistance to amikacin).

Subcluster I was characterized by the presence of a plasmid with a size of 10.7 kb and phenotypic resistance to penicillin, whereas subcluster II was characterized by having two plasmids of 10.7 and 1.3 kb and resistance to penicillin, ampicillin-sulbactam, and ciprofloxacin. Subcluster III featured two plasmids of 10.7 and 5.5 kb and resistance to penicillin. Finally, subcluster IV carried a plasmid of 1.0 kb and exhibited resistance to penicillin, amoxicillin - clavulanic acid, trimethoprim-sulfamethoxazole, ciprofloxacin, and aztreonam (Fig. 4). Considering the aforementioned, we deduced that these plasmids are associated with antibiotic resistance and that the plasmid with an approximate size of 10.7 kilobases was responsible for the resistance to penicillin, present in three of the four clusters with said resistance.

In this regard, Argente *et al*. [20] indicated that genes coding for extended-spectrum beta-lactamases (ESBL) are found in conjugative plasmids of variable sizes (7 to 160 kilobases) and tend to have a transfer frequency that varies from 10^{-7} to 10^{-2} per cell donor, thus facilitating the dissemination of resistance to different bacterial species. Jamborova *et al*. [21] identified different clones of *E. coli* with resistance to multiple antibiotics, such as quinolones and cephalosporins, associated with resistance genes that were found in 14 plasmids with variable size (3 to 200 kb). The plasmids identified in our study are likely to contain genes associated with resistance to the antibiotics tested; nevertheless, it is necessary to complement the information obtained with subsequent molecular analyses for the detection of the genes.

Amplification of genes linked to resistance to antibiotics in chromosomal DNA

In the 32 samples analyzed by gene amplification, 13 contained resistance genes including blaCTXM1, blaTEM, FosA, Mcr-1, and blaSHVI. Four of these 13 (namely, 1, 13, 27, and 31) had more than one resistance gene (**Suppl. 4**). The most common gene was blaTEM (37.5 %), followed by blaCTXM1, and blaSHVI (9.3 %). The least common genes were FosA and Mcr-1 (3.1 %). The remaining sample amplicons were observed but their size did not correspond to what was cited in the literature.

Nocua-Báez *et al*. [16] evaluated the presence of extended-spectrum beta-lactamases in *E. coli* from nine Colombian hospitals using molecular techniques and established that 6.9 % of the isolates expressed 15 blaTEM-1 and blaCTM-X-genes. In contrast, Hoang *et al*. [22] isolated strains of *E. coli* from healthy adults in Vietnam and noted the marked presence of blaCTX-M genes (blaCTX-M-9 and blaCTX-M-1), followed by blaTEM genes. Mahalingam *et al.* [17] analyzed the prevalence and transmissibility of the blaCTX-M, blaTEM, blaSHV, VIM, NDM, and OXA genes in

carbapenemase-producing *E. coli* in a tertiary care hospital in Southern India. They described that 95 % of the isolates showed beta-lactamase blaTEM and 62 % had the CTX-M type.

The profile of antibiotic-resistance genes in the *E. coli* chromosome from our study does not differ from that in other studies carried out in Colombia and in other countries; however, we highlight that this may me a conservative conclusion, because some samples yielded amplicons with a size different than expected. This may be due to variation within these resistance genes that was not evaluated in our work. This inference is supported by a report by Rada *et al*. [19] who reviewed the variants, geographical distribution, and molecular characterization of beta-lactamases collected in Colombia.

Antibiotic resistant *E. coli* represents a threat at the sanitary level since there are very few treatments to counteract the diseases caused by resistant microorganisms. In addition, the capacity for dissemination and transfer of resistance genes can challenge the treatment of diseases caused by these pathogens.

Amplification of genes from plasmid DNA linked to antibiotic resistance

Amplification of resistance genes from plasmid DNA was carried out in 16 isolates following total extraction of plasmid DNA. Genes such as blaTEM, blaSHV1, and blaCTXM1 were detected (**Suppl. 5**). The gene that predominated was blaTEM (37.5 %), followed by blaSHVI (25 %), and blaCTXM1 (6.25 %). None of the remaining isolates revealed amplificons for these genes according to product sizes reported in the literature. The determination of resistance genes in plasmids was carried out in total plasmid DAN from each of the isolates; the amplification process was not performed for each band of the profile, since the objective of our study was to verify the presence of these genes in chromosomal DNA and their association with plasmid DNA. We detected the blaTEM gene on plasmids of approximately 10 706 kb in size. Furthermore, this gene was found in six of the samples analyzed and it conferred resistance to penicillin and third-generation cephalosporins. In addition, we assumed that the reason why isolate 31 did not present the plasmid of 10 706 kb was because the blaTEM and blaCTXM1 genes were detected together and may be encoded by a larger plasmid.

The three plasmid antibiotic resistance genes mentioned above are the most common of its kind in studies carried out in other locations. For instance, Hoang *et al*. [22] reported that in the city of Ho Chi Minh in Vietnam, the most prevalent resistance genes were blaTEM and blaCTXM1, being directly

associated with plasmids with different molecular sizes. Furthermore, Guzman *et al*. [23] identified the genes blaTEM (65.4 %), blaCTX-M (34.6 %), and blaSHV (23.1 %) in multiresistant *E. coli* isolates from a community in Cumaná, Venezuela.

Isolate identity based on 16S rRNA sequencing

The 16S rRNA PCR amplification of the studied isolates resulted in amplicons of sizes about 1 500 bp (**Suppl. 6**). We employed the resulting 16S rRNA to query two databases (RDP and GenBank), taking into account the percentage of identity and the possible origin of the isolate (clinical sample). All of the 32 samples were identified as *E. coli*. The matching criteria included a coverage greater than 96 % and identity of at least 97 %, This result confirms the biochemical and morphological identification previously made.

All sequences were deposited in the *NCBI database*, with the following accession numbers: MN094112, MN094113, MN094114, MN094115, MN094116, MN094117, MN121153, MN094118, MN094119, MN094120, MN094121, MN094122, MN094123, MN094124, MN094125, MN094126, MN094127, MN094128, MN094129, MN094130, MN094131, MN094132, MN094133, MN094134, MN094135, MN094136, MN094137, MN121154,MN094138, MN121155, MN121156 and MN121157.

Two clusters are evidenced in the tree of *E. coli* samples of clinical origin (Fig. 5). In this tree, the ingroup, *was rooted* on an *E. coli* ATCC 25992 sequence and an antibiotic resistant *E. coli* sequence isolated in Colombia (28Eco12); the outgroup or external root consisted of a sequence from *Methanocaldococcus jannaschii*. In the first cluster, we observed that the evolutionary distance between the isolates is minimal because they all belong to the same species; however, there are variations in each isolate, which may be related to the environment in which each bacterial isolate develops and are generated by mutation (Fig. 5). In addition, we emphasize that all of the groupings formed in the tree are directly related to the pattern of resistance to antibiotics, as is the case of the grouping between isolates 16 and 23, which present a high similarity in the patterns of resistance and sensitivity to the antibiotics used. On the other hand, we also detected a monoclonal state between isolates 27 and 33 of *E. coli*, a fact that may be associated with the dissemination of resistance (P and CIP).

E. coli isolates from Colombia were grouped with an isolate exhibiting resistance to penicillin, ampicillin-sulbactam, and trimetropim sulfamethoxazole. This bacterium, according to Abril *et al*. [24], had antibiotic resistance genes such as aminoglycosides, beta-lactams, chloramphenicol, sulfonamides, trimetroprim, and tetracycline; so, it was

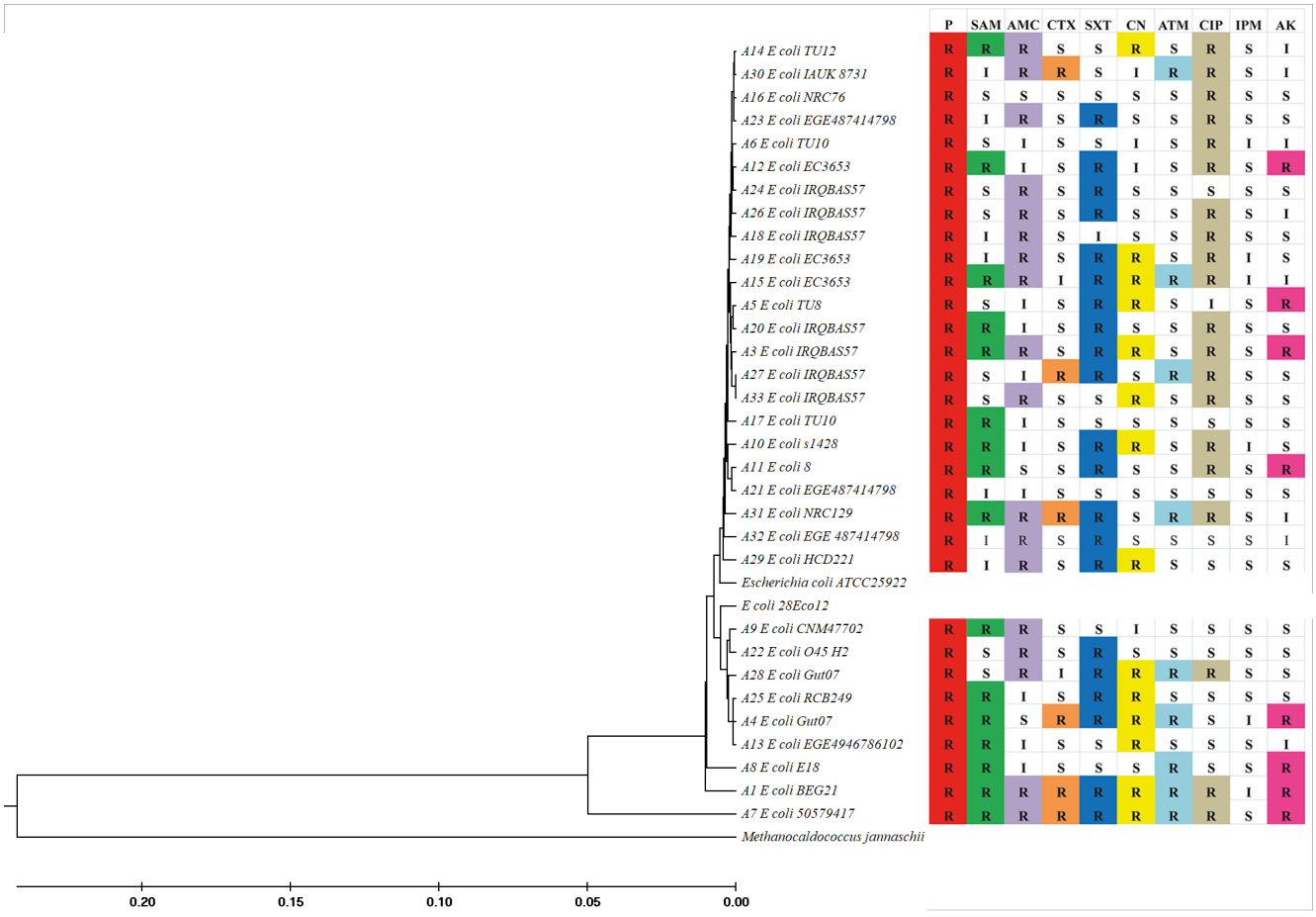

Figure 5. Phylogenetic tree of *E. coli* isolates. The phylogenetic tree was built using the Mega X 10.1 software and using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. Antibiotic resistance is color-coded. Red (resistance to Penicillin), green (resistance to amoxicillin), orange (resistance to cefotaxime), blue (resistance to trimethoprim-sulfamethoxazole), yellow (resistance to gentamicin), light blue (resistance to aztreonam), light brown (resistance to ciprofloxacin) and fuchsia (resistance to amikacin).

taken into account for comparison's sake. No further sequences were added for comparison since there are very few reports of *E. coli* 16S rRNA genes directly associated with antibiotic resistance.

Amplification of intergenic sequences of repetitive consensus of enterobacteria (enterobacterial repetitive intergenic consensus-PCR, *ERIC-PCR*)

Fig. 6 shows the different profiles generated by the *ERIC-PCR* technique. The samples presented bands with sizes ranging between 4.7 kb with a

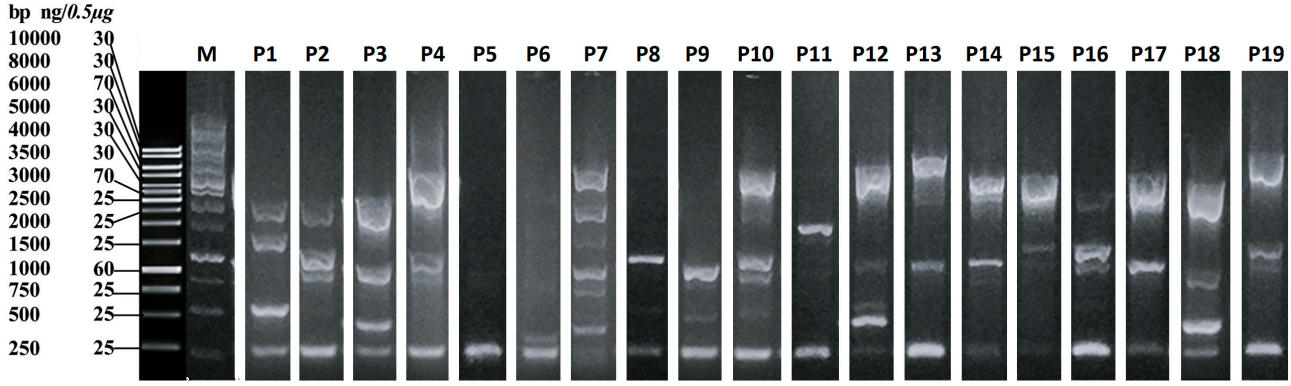

Figure 6. Verification of the *ERIC-PCR* amplification. In the first lane, the molecular marker 1 kb is observed; lanes 2 to 19, differential profiles of *E. coli* isolates. P: profiles.

total of 20 profiles evidenced and a 0.2-kb band was shared by all the isolates. The analysis showed four genetically identical clonal states with a similarity percentage of 100 %. The identical groupings showed a direct relationship with resistance to penicillin, and each group presented its own pattern of sensitivity to antibiotics. Clonal state I showed sensitivity to trimetropin-sulfamethoxazole, cefotaxime, and ciprofloxacin; clonal state II exhibited sensitivity to aztreonam; state III displayed sensitivity to aztreonam and cefotaxime; and state IV showed sensitivity to amikacin (**Fig. 7**).

ERIC-PCR enables the analysis of the clonal relation among microorganisms, facilitating the tracking of their dissemination and population identification. We were also able to determine four predominant clusters of *E. coli* isolates, which share characteristics of resistance to specific antibiotics in a hospital environment. We observed that cluster II had a greater number of isolates, so these clones may be responsible for the spread of resistance. However, it is necessary to carry out analytical studies to estimate the true impact of clonal dissemination. On the other hand, low similarity values in the other groups can be attributed to genetic events such as mutations, insertions or deletions that occur spontaneously and are part of the evolution of each population [12, 23, 25].

Considering both the microbiological and molecular characterization of *E. coli* isolates, we can state that there is ample variation at the genetic (*ERIC-PCR*, plasmid profile, and resistance genes) and phenotypic (resistance profile) levels among the 32 samples analyzed. Our research can be considered as an indicative study, which can be extended to other health institutions. In this sense, we would like to note that new resistance variants are constantly

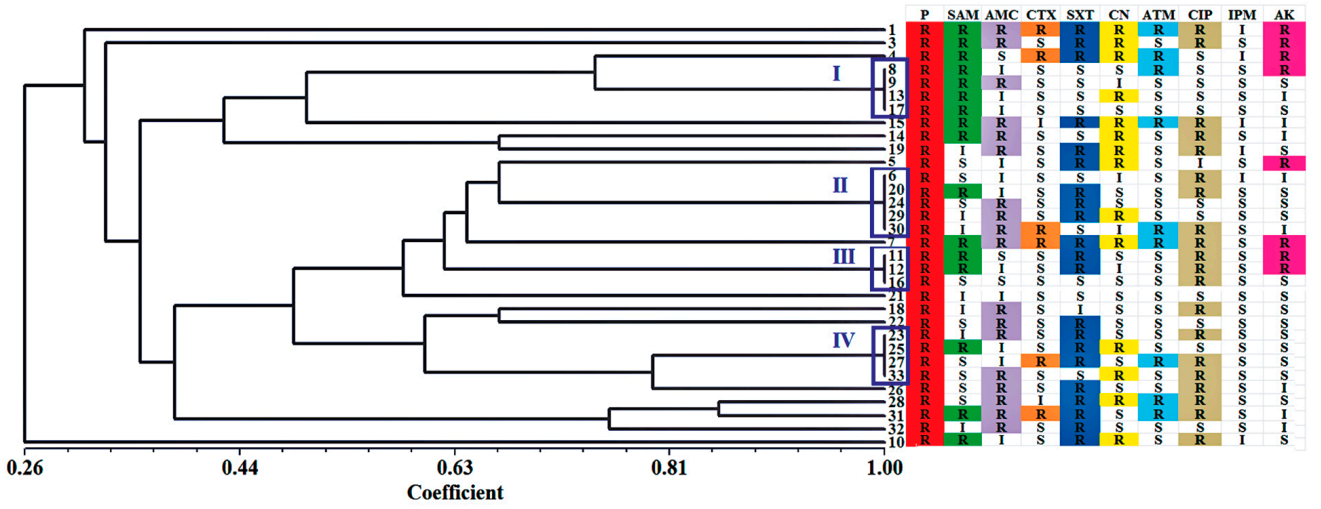

Figure 7. Dendrogram of the *ERIC-PCR* profile of the *E. coli* isolates. Built using the NTSYS Spc 2.1 software and using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. Antibiotic resistance is color-coded. Red (resistance to Penicillin), green (resistance to amoxicillin), orange (resistance to cefotaxime), blue (resistance to trimethoprim-sulfamethoxazole), yellow (resistance to gentamicin), light blue (resistance to aztreonam), light brown (resistance to ciprofloxacin) and fuchsia (resistance to amikacin). Clonal isolates (marked in blue boxes) are shown in each of the clusters with a similarity coefficient of 100 %.

being generated and traditional treatments are losing effectiveness against these microorganisms. Therefore, it is important to establish surveillance and control plans against inadequate antibiotic use. Since antibiotic resistance is chiefly triggered by its sustained and improper use, it is also important to establish successful mitigation strategies to counter environmental resistance.

Conclusions

Clinical isolates of *E. coli* showed marked resistance to most antibiotics tested in this study (P, SXT, CIP, AMX, AMC, and GN). Thirteen isolates of *E. coli* had resistance genes: blaCTXM1, blaTEM, FosA, Mcr -1, and blaSHVI on the chromosomal DNA, indicating that these virulence factors are being integrated into the genetic information of the bacteria.

The clonal states generated by the *ERIC-PCR* marker demonstrate identical characteristics among the bacterial isolates. Sixteen isolates of *E. coli* presented plasmid profiles of one to five bands with molecular sizes of 0.9 to 18.7 kb. These fragments were directly associated with the resistance genes: blaTEM, blaSHV1, and blaCTXM1.

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Conflict of interests

The authors declare having no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent arrangements), or none (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript. The authors declare that this work does not entail any conflict of interests.

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Caracterización de *Escherichia coli* resistente a antibióticos asociada con infecciones del tracto urinario en el sur de Colombia

Resumen: La resistencia bacteriana a antibióticos es uno de los mayores problemas médicos en el mundo. Una de las bacterias de más alta prioridad es *E. coli*, ya que presenta diferentes mecanismos de resistencia y algunas de sus cepas han desarrollado resistencia a los antibióticos betalactámicos. Hemos caracterizado 32 aislados bacterianos resistentes a antibióticos a partir de casos confirmados de infecciones del tracto urinario en un grupo de pacientes en Nariño, sur de Colombia. Se realizaron descripciones macro y microscópicas de los 32 aislados clínicos. Se efectuaron perfiles de resistencia y caracterización bioquímica y molecular (mediante secuenciación del gen 16S ARNr, *ERIC-PCR* y genes de resistencia). Todos los aislados fueron identificados como *E. coli* y presentaron resistencia a betalactámicos, aminoglucósidos y fluoroquinolonas. Esta resistencia se relacionó con los plásmidos que llevan los genes de resistencia blaTEM, blaSHV1 y blaCTXM1. Hubo diferencias significativas entre las proporciones de resistencia de las muestras (valor de p: 0.0000), principalmente a penicilina, cefotoxina e imipenem. Mediante ERIC-PCR se evidenciaron cuatro estados clonales, que corroboran un grado de diferenciación genética dentro del grupo de aislados estudiados. La resistencia a los antibióticos observada en los aislados está asociada con genes de resistencia presentes en el cromosoma bacteriano y en los plásmidos.

Palabras clave: antibióticos; *Escherichia coli*; infecciones urinarias; plásmidos y resistencia.

Caracterização de *Escherichia coli* resistente a antibióticos associada com infecções do trato urinário no sul da Colômbia

Resumo: A resistência bacteriana a antibióticos é um dos maiores problemas médicos no mundo. Uma das bactérias de maior prioridade é *E. coli*, uma vez que apresenta diferentes mecanismos de resistência e algumas de suas cepas vem desenvolvendo resistência aos antibióticos betalactâmicos. Caracterizaram-se 32 isolamentos bacterianos resistentes a antibióticos a partir de casos confirmados de infecções do trato urinário em um grupo de pacientes de Nariño, sul de Colômbia. Realizaram-se descrições macro e microscópicas dos 32 isolamentos clínicos. Efetuaram-se perfis de resistência e caracterização bioquímica e molecular (via sequenciação do gen 16S ARNr, *ERIC-PCR* e genes de resistência). Todos os isolamentos foram identificados como *E. coli* e presentaram resistência a betalactâmicos, aminoglicosídeos e fluoroquinolonas. Esta resistência se relacionou com os plasmídeos que presentam os genes de resistência. blaTEM, blaSHV1 e blaCTXM1. Houve diferenças significativas entre as proporções de resistência das amostras (valor de p: 0,0000), principalmente a penicilina, cefotoxina e imipenem. Por meio de ERIC-PCR se evidenciou quatro estados clonais, que corrobora um grau de diferenciação genética dentro do grupo de isolamentos estudados. A resistência aos antibióticos observada nos isolamentos está associada com genes de resistência presentes no cromossoma bacteriano e nos plasmídeos.

Palavras-chave: antibióticos; *Escherichia coli*; infecciones urinárias; plasmídeos; resistência.

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