

ORIGINAL RESEARCH

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Differential molecular approach and ESBL detection from *Klebsiella pneumoniae* and *Escherichia coli* isolated from the supraglottic region of patients undergoing mechanical ventilation in an intensive care unit

Aproximación molecular diferencial y detección de BLEES a partir de Klebsiella pneumoniae y Escherichia coli aisladas de la región supraglótica de pacientes sometidos a ventilación mecánica en una unidad de cuidados intensivos

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| Abstract |

Introduction: Given their ability for colonizing the supraglottic region, desiccation tolerance, resistance to β -lactam antibiotics, and adherence to both inert surfaces and epithelial cells, *Klebsiella pneumoniae* and *Escherichia coli* are potentially pathogenic microorganisms for patients undergoing mechanical ventilation in an intensive care unit (ICU).

Objective: To perform a molecular characterization and detection of extended spectrum β -lactamases (ESBL) in *K. pneumoniae* and *E. coli* strains isolated from the supraglottic region of patients undergoing mechanical ventilation in an ICU.

Materials and methods: A descriptive study was conducted in 18 isolates. Disk diffusion technique was used for detecting ESBL-producing bacteria. Molecular characterization was made by BOX-PCR technique, while ESBL production was confirmed by testing the isolates against cefotaxime and ceftazidime, alone and in combination with clavulanic acid.

Results: a *K. pneumoniae* strain and another *E. coli* strain were confirmed as ESBL producers. A divergence greater than 50% was observed in most of the strains; besides non-infectious origin strains resistant to third generation cephalosporins were found.

Conclusion: The polyclonality found in this study might indicate that most of the strains belong to each patient's microbiota.

Keywords: Beta-lactamases; Gram-Negative Aerobic Bacteria; Mechanical Ventilation; Antimicrobial Drug Resistances; Intensive Care Unit (MeSH).

| Resumen |

Introducción. Dada su capacidad para colonizar la región supraglótica, tolerar desecación, resistir los antibióticos β -lactámicos y adherirse tanto a superficies como a células epiteliales, la *Klebsiella pneumoniae* y la *Escherichia coli* son microorganismos potencialmente patógenos para los pacientes de la unidad de cuidados intensivos (UCI) sometidos a ventilación mecánica.

Objetivo. Realizar la caracterización molecular y la detección de β -lactamasas de espectro extendido (BLEES) a cepas de *K. pneumoniae* y *E. coli* aisladas de la región supraglótica de pacientes internados en UCI y sometidos a ventilación mecánica.

Materiales y métodos. Estudio descriptivo realizado en 18 aislamientos. Se utilizó la técnica de difusión en disco para detectar bacterias productoras de BLEES. La caracterización molecular se realizó mediante la técnica de BOX-PCR y la producción de ESBL fue confirmada mediante la prueba con cefotaxima y ceftazidima, solas y combinadas con ácido clavulánico.

Resultados. Una cepa de *K. pneumoniae* y otra de *E. coli* resultaron productoras de BLEES. La mayoría de cepas presentaron una divergencia superior al 50%, evidenciándose, además, cepas de origen no infeccioso resistentes a cefalosporinas de tercera generación.

Conclusión. La policlonalidad encontrada podría indicar que la mayoría de las cepas pertenecen a la microbiota de cada paciente.

Palabras clave: Inhibidores de beta-Lactamasas; Bacterias aerobias Gramnegativas; Ventilación mecánica; Resistencia microbiana a antibióticos; Unidad de cuidados intensivos; resistencia bacteriana a múltiples medicamentos (DeCS).

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Introduction

The admission of patients to the intensive care unit (ICU) has an effect on the frequency of infections caused by strains of multi-resistant Gram-negative bacilli (MRGN). Some risk factors increase the susceptibility to MRGN infections, including those caused by extended spectrum beta-lactamases (ESBL) producing strains. Some of these risk factors include immunosuppressive treatments in diseases such as cancer (1), transplant interventions (2), the implementation of central or urinary catheters (3), hemodialysis procedures (4), and mechanical ventilation devices. (5)

Klebsiella pneumoniae and *Escherichia coli* are Gram-negative bacilli that are present in the intestinal tract of mammals, and their colonization rates are trebled in hospital environments in a direct proportion to the length of the stay. Furthermore, their colonization degree has been associated with the selective pressure exerted by antibiotics on intestinal microbiota. (6,7)

These microorganisms are potentially pathogenic for ICU patients undergoing mechanical ventilation since, after colonizing the supraglottic region, they adhere to both inert surfaces and epithelial cells and trigger ventilator-associated pneumonia (VAP) in order to resist desiccation and β -lactam antibiotics respectively. (8,9)

Currently, four pathogenic pathways for VAP to occur have been described: by aspirating secretions through the oropharynx, by contiguity, by entering into contact with blood (hematogenous route), and through breathing circuits. Aspiration of secretions through the oropharynx is the most common pathway (10), therefore biofilms containing respiratory pathogenic microorganisms from the supraglottic region constitute a source of pneumonia associated with health care.

Microbiological analysis by molecular methods performed on the tongue and from bronchoalveolar lavage has evidenced the presence of a several bacterial species. (11) Some tests have suggested that these microorganisms are able to act as pathogens that affect the lower respiratory tract. (11) The isolation of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter spp*, and enterobacteria from dental plaque in patients with pneumonia associated with health care, indistinguishable of isolates obtained from bronchoalveolar lavage, reinforces this idea. (11)

This study was made from the data obtained in a prior research where the effectiveness of chlorhexidine mouthwash was compared to toothpaste mouthwash in terms of decontamination of the bacterial microbiota in the supraglottic region. In addition, reduction of the supraglottic region bacterial microbiota in patients undergoing mechanical ventilation at the ICU was evaluated. After 72 hours in the ICU, colonization of the oropharynx by normal microbiota was reduced, while pathogenic microbiota increased, being *K. pneumoniae* and *E. coli* the most common microorganisms. (12) Some of these strains were preserved in order to determine, as it is proposed here, their susceptibility against antibiotics, as well as their molecular profiles.

Considering the above mentioned, this study focused on the molecular characterization and detection of ESBL-producing *K. pneumoniae* and *E. coli* strains isolated from the supraglottic region of patients undergoing mechanical ventilation in an ICU.

Tovar OL, Estrada GI, Florián MC, Uribe A, Marulanda CA, Corpas-Iguarán E, et al. [Aproximación molecular diferencial y detección de BLEES a partir de *Klebsiella pneumoniae* y *Escherichia coli* aisladas de la región supraglótica de pacientes sometidos a ventilación mecánica en una unidad de cuidados intensivos]. Rev. Fac. Med. 2018;66(4):581-7. English. doi: <http://dx.doi.org/10.15446/revfacmed.v66n4.63424>.

Materials and methods

Type of study and sample unit

A descriptive study was carried out. Microorganisms were preserved in the Collection of Microorganisms of Universidad Católica de Manizales (CM-CUM).

The data was collected from a prior study conducted in patients undergoing mechanical ventilation at the ICU of a hospital in Manizales, Colombia, between 2013 and 2014. The sample consisted of 18 isolates of *K. pneumoniae* and 7 isolates of *E. coli* obtained from the supraglottic region of said patients. In addition, 5 isolates of *K. pneumoniae* obtained from blood cultures, external secretion and peritoneal fluid of patients admitted to the same ICU, but not included in the first study, were also considered for molecular analysis.

Assessment of response to antimicrobials and ESBL production

The preserved strains were initially grown on nutrient agar and were subsequently inoculated on a selective medium (MacConkey agar). These preserved strains were confirmed in terms of gender and species through the automated system VITEK® 2 compact (BioMérieux) at the UCM laboratory.

After confirming the identification, antimicrobial susceptibility was determined by means of the disk diffusion method on selective medium (Mueller Hinton Agar), in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI), (13) including the following antibiotics for detecting possible ESBL-producing strains: aztreonam, cefotaxime, cefotetan, ceftazidime and ceftriaxone.

For preparing the inoculum, 4 to 5 colonies of similar morphology were selected from each culture and were homogenized in 5mL of saline solution, while the resulting turbidity in the solution was adjusted by using sterile saline solution until a density equivalent to 0.5 on the McFarland standard was achieved. Within 15 minutes after adjusting the inoculum, the spread plate technique on a Mueller Hinton agar was performed by using a sterile swab, and then, antibiotic disks were placed on the surface of the inoculated agar through sterile forceps. The disks were distanced at a 24mm average length. Afterwards, plates were incubated at 37°C for 16 to 18 hours. Finally, diameters of inhibition zones were measured according to the CLSI criteria. (13) To confirm ESBL-producing strains, double-disk diffusion method was used in accordance with the CLSI standard and the following breakpoints: cefpodoxime (≤ 22 mm), aztreonam (≤ 27 mm), ceftazidime (≤ 22 mm), cefotaxime (≤ 27 mm) or ceftriaxone (≤ 25 mm) (13).

ESBL production by the strains studied was confirmed through growth measurement against cefotaxime and ceftazidime alone and in combination with clavulanic acid. A ≥ 5 mm difference between the diameter of the disk where ceftazidime + clavulanic acid were used and the disk where only ceftazidime was used, or between the disk where cefotaxime + clavulanic acid were applied and the one where only cefotaxime was used, was considered as ESBL production.

Genotyping by BOX-PCR system

Bacterial DNA was extracted by using the UltraClean® Blood DNA Isolation Kit (NON-SPIN), which was provided by MOBIO laboratories, Inc. Subsequently, the DNA obtained was stored in Eppendorf tubes at -20°C until its amplification by polymerase chain reaction (PCR) was done.

BOX-PCR typing was performed with the AR1-5'-CTACGGCAAGGCGACGCTGACG-3' primer under the following conditions: 100ng of DNA were amplified in a mini thermal cycler (Bio Rad®) and were arranged in a reaction volume of 25µL, (14,15) which contained 0.2mM of dNTPs, 2mM of MgCl₂, 1.5µM of primer, 0.10mg/mL of BSA, 10% of DMSO and 1U/µL of Taq DNA polymerase (BIOLINE®). For the BOX-PCR amplification, first the DNA was denatured for 5 minutes at 95°C and then underwent 30 denaturation cycles (92°C, 30 sec), an association cycle (60°C, 1 min), and one extension cycle (65°C, 8 min), which was followed by a final extension cycle at 65°C for 8 min. Amplified products were separated by electrophoresis on 2% agarose gel with 1.0 X TBE buffer, for 3.5 h at 4.6 V/cm. Banding patterns were recorded in photos (14,15).

Finally, visual inspection of electrophoretic profiles was done and matrices of presence and absence of bands for the *K. pneumoniae* and *E. coli* isolates were constructed by using Microsoft Office Excel software.

Statistical analysis

The analysis of the matrices was done by using NTSYSpc 2.2 version software. A dendrogram with the unweighted pair group method with arithmetic averages algorithm and Jaccard similarity coefficient was created.

The similarity coefficient (S) was obtained based on the following equation: $S = 2 * N_{ab} / a + b$, where N_{ab} is the total number of similar bands between 2 isolates and $a + b$ is the total sum of the number of bands of isolates a and b.

Ethical considerations

The microorganisms used in this study were collected in a prior research that was approved by the Research Ethics Committee of the Universidad Católica de Manizales on April 18, 2012, this approval was updated in September 2018 by this committee, it should be noted that due to the university policies the approvals issued by said ethics committee are not numbered. In addition, participants' informed consent was duly obtained in said research.

Results

K. pneumoniae response to antimicrobials and ESBL production

Out of the 18 *K. pneumoniae* strains analyzed, sensitivity to aztreonam, cefotaxime, ceftriaxone, cefotetan and ceftazidime was observed in 11 (61%), while the 7 remaining strains (39%) were resistant to these antibiotics. Intermediate sensitivity was not observed in any of the isolations (Figure 1).

Then, 12 potentially ESBL-producing strains were selected and they were assigned the following GIBI codes: 104, 106, 162, 157, 335, 344, 330, 338, 340, 332, 331, and 343. Through the disk diffusion technique, an ESBL test was done for each of the strains, the results are as follows: 11 negative isolates and 1 ESBL-producing strain (Table 1).

E. coli response to antimicrobials and ESBL production

Out of 7 *E. coli* strains analyzed, 6 (86%) showed sensitivity to aztreonam, cefotaxime, ceftriaxone, cefotetan, and ceftazidime. Intermediate sensitivity was not observed in any of the isolations (Figure 2).

Later, 4 potentially ESBL-producing strains were selected and were given the following GIBI codes: 160, 169, 171, and 334. Results after performing the ESBL production confirmatory test were as follows: negative for 3 isolates, and positive for 1 strain (Table 2).

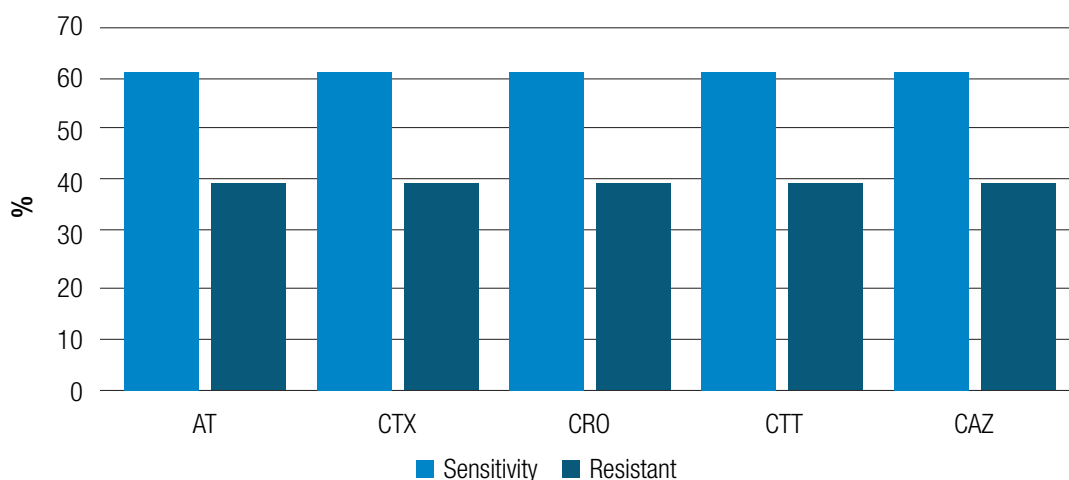


Figure 1. Percentage of sensitivity of *Klebsiella pneumoniae* strains against different antibiotics.

At: Aztreonam; CTX: Cefotaxime; CRO: Ceftriaxone; CTT: Cefotetan; CAZ: Ceftazidime.

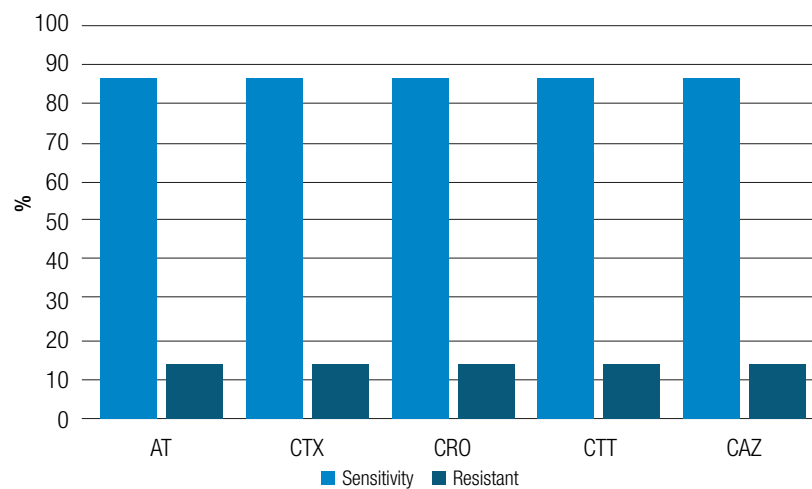
Source: Own elaboration.

Table 1. ESBL production test performed on the 12 selected *Klebsiella pneumoniae* strains.

GIBI CODES	CTX	CTX-CA	CAZ	CAZ-CA
104	31mm	31mm	28mm	28mm
106	30mm	30mm	30mm	31mm
162	33mm	33mm	30mm	30mm
157	31mm	32mm	28mm	29mm
335	11mm	16mm	Resistant	10mm
344	28mm	29mm	28mm	29mm
330	<10mm	10mm	Resistant	Resistant
338	12mm	13mm	<10mm	10mm
340	23mm	25mm	18mm	21mm
332	<10mm	11mm	Resistant	Resistant
331	10mm	14mm	Resistant	<10mm
343	<10mm	11mm	Resistant	<10mm

CTX: Cefotaxime; CAZ: Ceftazidime; CA: Clavulanic acid.

Source: Own elaboration.

**Figure 2.** Percentage of sensitivity of *Escherichia coli* strains against different antibiotics.

At: Aztreonam; CTX: Cefotaxime; CRO: Ceftriaxone; CTT: Cefotetan; CAZ: Ceftazidime.

Source: Own elaboration.

Table 2. ESBL production test performed on the 4 selected *Escherichia coli* strains.

GIBI CODES	CTX	CTX-AC	CAZ	CAZ-AC
160	30mm	31mm	27mm	28mm
169	30mm	30mm	28mm	29mm
171	Resistant	20mm	15mm	21mm
334	28mm	28mm	27mm	28mm

CTX: Cefotaxime; CAZ: Ceftazidime; CA: Clavulanic acid.

Source: Own elaboration.

Electrophoretic profile of *K. pneumoniae* strains

Regarding the results obtained through the BOX-PCR fingerprinting technique, out of 23 *K. pneumoniae* isolates, 2 groups with a divergence greater than 50% were observed. In the first group, constituted by 12

isolates, 3 subgroups were identified: subgroup 1, with isolates 62 and 344; subgroup 2, with isolates 72 and 331, and subgroup 3, where isolates 73, 104, 102, 92, 332, 335, 330, and 112 were found. It is noteworthy that isolate 112 had a divergence greater than 50% in comparison with the other isolates of this subgroup. In addition, a 100% similarity between

isolates 73 and 104 was observed, as well as between isolates 92, 332, and 335, while similarity among other isolates was less than 90% (Figure 3).

On the other hand, in the second group, made up by 11 isolates, 3 subgroups were found: isolates 79 and 338 in subgroup 1, isolate

103 in subgroup 2, and strains 164, 168, 106, 157, 163, 162, 340, and 343 in subgroup 3. A 100% similarity between isolates 157 and 163 was observed, while similarity among other isolates in the remaining subgroups was less than 90% (Figure 3).

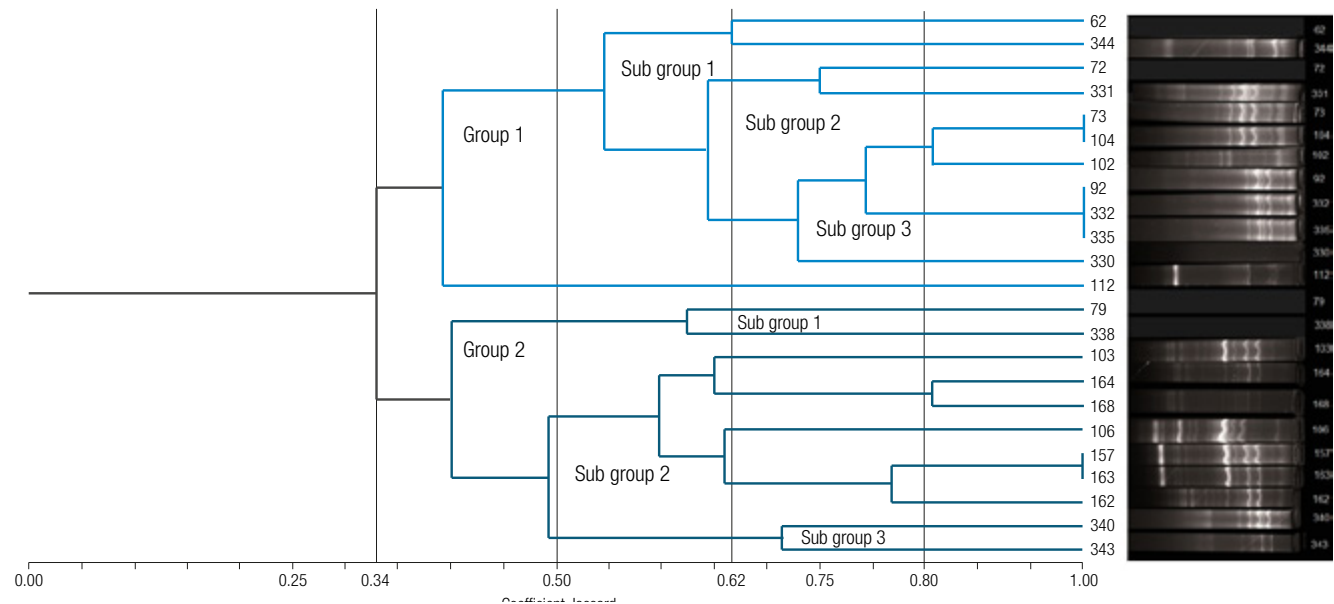


Figure 3. Cluster analysis to determine similarity among *Klebsiella pneumoniae* strains and electrophoretic profiles according to the BOX-PCR test. Source: Own elaboration.

Electrophoretic profile of *E. coli* strains

According to the results obtained after performing the BOX-PCR test, a divergence greater than 50% in the 7 isolates was found. A

92% similarity between isolates 159 and 160 was observed, while for isolates 161 and 344 a 91% similarity was reported (Figure 4).

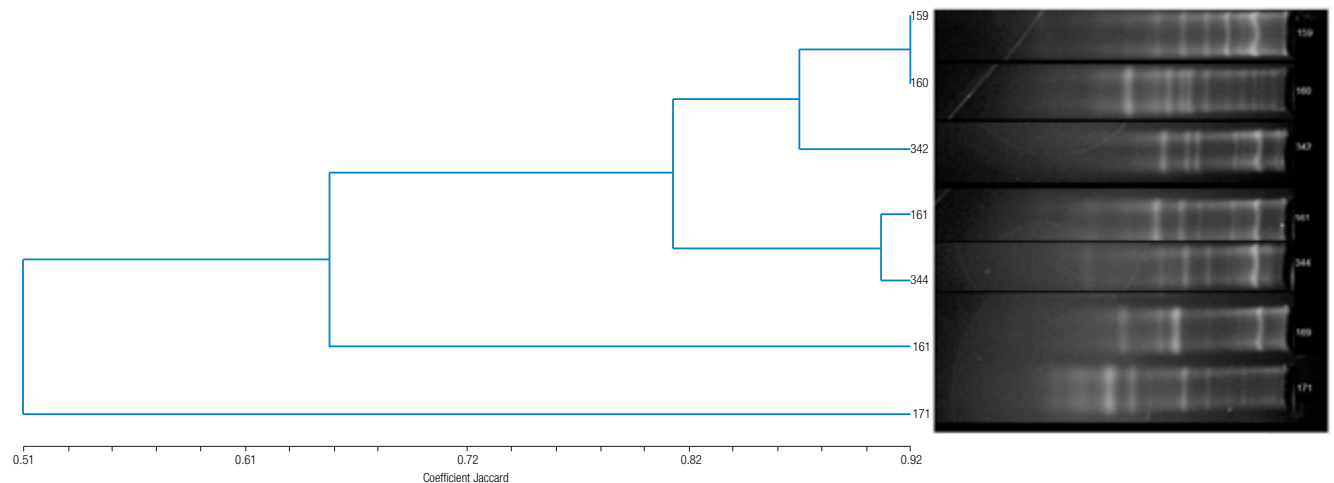


Figure 4. Cluster analysis to determine similarity among *Escherichia coli* strains and electrophoretic profiles according to the BOX-PCR test. Source: Own elaboration.

Discussion

In this study one type of ESBL-producing strain was identified. In Colombia, higher percentages of ESBL-producing *K. pneumoniae* and *E. coli* strains have been reported. For example, in a study conducted in 22 *K. pneumoniae* isolates obtained from patients with a hospital-acquired infection, 15 were resistant to ceftazidime, cefotaxime, ceftriaxone and aztreonam. Besides, all the 15 strains were confirmed as ESBL-

producing. (16) Likewise, another research conducted on 144 isolates of *E. coli* and *K. pneumoniae*, obtained from patients staying at several hospitalization services, reported that 48.6% of the total sample were resistant to any cephalosporin, while 25.6% of the *E. coli* and 48.4% of the *K. pneumoniae* strains were ESBL-producing. (17) Another study carried out in a hospital in Valledupar (Colombia) reported a 12.3% ESBL-producing enterobacteria, with *E. coli* and *K. pneumoniae* as the most frequent strains (55.6% and 23.2%, respectively). (18)

Prior studies in Colombia have focused on strains with an infectious origin, while in this research isolates were obtained from the microbiota of the supraglottic region. Considering their origin, the isolates were not expected to be resistant to third generation cephalosporins or ESBL producers, which explains why the results obtained here indicate that the prevalence of ESBL-producing strains was lower than those reported in previous studies. Furthermore, resistance to third generation cephalosporins by strains from the supraglottic region may imply that there is a possibility of other resistance mechanisms such as alteration of target sites related to the penicillin-binding protein (PBP), reduction of porin-mediated outer membrane permeability, and efflux pumps. (9)

It has also been reported that dissemination in the hospital environment occurs mainly because of the presence of strains of endogenous origin that are associated with each patient's microbiota. (16) Moreover, the resistance observed in the strains obtained from the supraglottic region could be explained by the fact that treatments against various infections are made through empiric therapy, namely, without knowing the etiology and antimicrobial susceptibility of the pathogen involved in the infection. Likewise, other inappropriate practices such as self-medication promote the development of resistance mechanisms. When these factors come together, any type of resistance to be acquired will have an effect on the effectiveness of antimicrobial treatments, thus generating therapeutic limitations in hospitals.

On the other hand, the 100% genotypic similarity between some of the strains studied here suggests a common intrahospital contamination source, as well as some cross contamination events, since patients' dates of admission and stays at the ICU were different.

The results of this study indicate the need for controlling ESBL-producing strains from the supraglottic microbiota of patients referred to the ICU. One strategy to control said strains is to use routine surveillance cultures, which also includes an educational intervention for the staff involved. (19) Regarding this approach, it is necessary to raise the awareness on the possibility of using microbial culture on a regular basis as an alert tool, especially if there is enough evidence to consider that some patients may be hosts of ESBL-producing strains prior to their admission to the hospital or the ICU.

In this sense, Boyer *et al.* (20) reported the need to eliminate circulating strains in the environment. Therefore, monitoring potential sources of contamination that are usually underestimated, such as sinks, is an appropriate practice, for sometimes these sources may contain ESBL-producing bacteria and their elimination helps reducing the incidence of multiresistant strains in any ICU. (21) In that regard, the installation of self-disinfecting siphons in sinks of ICUs has been proved to remove biofilm formation and reduce transmission of ESBL-producing Gram negative bacteria. (22)

Experts on this matter agree that genotyping by BOX-PCR technique offers an adequate discriminatory power, as well as reproducibility, and that is a fast typing alternative to get fast results. Furthermore, it requires less investment in comparison with other techniques such as gel electrophoresis by pulsed fields (PFGE) and DNA sequencing. (3) Somehow, a more efficient approach involving the identification of differential resistance genes could include matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), considering the reduced times it requires and the possibility of obtaining specific mass spectra of genus and species it offers. (23)

Conclusions

A greater than 50% divergence in the molecular profiles among the strains groups of *K. pneumoniae* and among those of *E. coli* was

determined. This polyclonality indicates that most of the strains belonged to the microbiota of each patient.

The isolation of *K. pneumoniae* and *E. coli* resistant to third generation cephalosporins and ESBL-producing strains in the ICU had as its main source the microbiota of each patient. Cases of genotypic similarity of 100% suggest a common intrahospital contamination source. These cases involved cross contamination events, since the date of admission and the time of permanence of patients in the ICU were different.

Considering their pathogenicity mechanisms, the presence of *E. coli* and *K. pneumoniae* resistant to β -lactam antibiotics strains in the supraglottic region could trigger persistent infections such as VAP, which is considered as a major financial challenge for the maintenance of public health.

Conflicts of interest

None stated by the authors.

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