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ORIGINAL RESEARCH

Molecular characteristics of antibiotic-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates obtained from urine samples of patients with urinary tract infection in Lima and Callao, Peru

Características moleculares de aislamientos de Escherichia coli y Klebsiella pneumoniae resistentes a antibióticos obtenidos de muestras de orina de pacientes con infección del trato urinario en Lima y Callao, Perú

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Palabras clave: *Escherichia coli; Klebsiella pneumoniae;* Farmacorresistencia Bacteriana; Resistencia betalactámica; Genes; Perú (DeCS).

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Abstract

Introduction: Urinary tract infections (UTI) are the second most frequent disease caused by bacteria, mainly *Escherichia coli* and *Klebsiella pneumoniae*. Furthermore, the emergence of multidrug-resistant extended-spectrum β-lactamases (ESBL)-producing bacteria is a serious public health issue.
Objective: To describe the molecular characteristics of ESBL-producing *E. coli* and *K. pneumoniae* isolates obtained from urinary samples of Peruvian patients with UTI.
Materials and methods: Retrospective, descriptive, cross-sectional study, in which 118 isolates obtained from urine cultures of patients with UTI treated at 2 hospitals located in the province of Lima and 1 in the province of Callao between April and August, 2019, were analyzed. A MicroScan™ automated system and a conventional polymerase chain reaction (PCR) test were used to identify resistance profiles and detect ESBL genes, respectively.
Results: All the bacteria isolated in the 3 hospitals were multi-drug resistant (105 *E. coli* and 13 *K. pneumoniae*). Coexistence of ESBL genes (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$) was observed in 32.20% of the isolates (28.57% of *E. coli* and 61.53% of *K. pneumoniae* isolates). Coexistence of 2 and 3 genes was found in 12.71 % and 21.18 % of isolates, respectively. In addition, *bla*$_{TEM}$ was the ESBL gene most frequently expressed in the isolates (45.76%).
Conclusions: Multiple drug resistance was found in all isolates analyzed. Additionally, coexistence of ESBL genes was observed in almost one third of the isolates, showing that antibiotic resistance is a real problem in public hospitals in the provinces of Lima and Callao.

Resumen

Introducción. Las infecciones del tracto urinario (ITU) son la segunda enfermedad más frecuente causada por bacterias, principalmente por *Escherichia coli* y *Klebsiella pneumoniae*; además, la aparición de bacterias multidrogorresistentes productoras de betalactamasas de espectro extendido (BLEE) representa un serio problema de salud pública.
Objetivo. Describir las características moleculares de aislamientos de *E. coli* y *K. pneumoniae* productoras de BLEE obtenidos de muestras de orina de pacientes peruanos con ITU.
Materiales y métodos. Estudio retrospectivo, transversal y descriptivo. Se analizaron 118 aislamientos de urocultivos de pacientes con ITU atendidos en 2 hospitales de la provincia de Lima y 1 de la provincia del Callao procesados entre abril y agosto del 2019. Los perfiles de resistencia se identificaron utilizando el sistema automatizado MicroScan™ y para la detección de los genes BLEE se empleó una prueba de reacción de cadena de la polimerasa convencional.
Resultados. El 100% de las bacterias aisladas en los tres hospitales fueron multidrogorresistentes (105 de *E. coli* y 13 de *K. pneumoniae*). La coexistencia de genes BLEE (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$) se observó en 32.20% de los aislamientos (28.57% de los de *E. coli* y 61.53% de los de *K. pneumoniae*), hallándose coexistencia de 2 genes y 3 genes en 12.71% y 21.18%, respectivamente; además, *bla*$_{TEM}$ fue el gen BLEE más frecuentemente expresado en los aislamientos (45.76%).
Conclusiones. Se halló multidrogoresistencia en todos los aislamientos analizados. Además, se observó coexistencia de genes BLEE en casi un tercio de todos los aislamientos, lo que evidencia que la resistencia a los antibióticos es una problemática real en los hospitales públicos de las provincias de Lima y Callao.

Introduction

Urinary tract infections (UTI) have become one of the most important infectious diseases, being found both in hospitals and in the community, thus resulting in a growing number of cases and increasing morbidity and mortality.[1] The most frequent causative agents of UTI are from the *Enterobacteriaceae* family, with *Escherichia coli* causing 70-95% of all cases.[2]

The selective pressure on bacteria caused by poor medical prescription or inadequate use of antibiotics has led to a considerable increase in bacterial resistance to antibiotics, even to the point where clinical treatments have lost effectiveness.[3] Antimicrobial resistance is a public health concern that is critically on the rise, so much so that if this trend does not change, it is estimated that the death rate caused by antimicrobial-resistant bacteria will be 10 million by 2050, which will have both social and economic implications and will jeopardize the development of countries.[4]

Extended-spectrum beta-lactamases (ESBLs) cause resistance to most beta-lactam antibiotics, including the penicillin families; first-, second, and third-generation cephalosporins; and monobactams.[5] The production of these enzymes is encoded by the expression of several bacterial resistance genes referred to as ESBL genes, which, when expressed, secrete enzymes that hydrolyze the endocyclic peptide bond of the beta-lactam ring of antibiotics and, consequently, deactivate their bactericidal action.[5,6]

The genes that typically encode for ESBLs are from the temoniera (TEM), sulfhydryl variable (SHV) and cefotaxime (CTX-M) families. Moreover, CTX-M type beta-lactamases have been identified as the most prevalent worldwide.[7]

In view of the above, the objective of the present study was to describe the molecular characteristics of ESBL-producing *E. coli* and *Klebsiella pneumoniae* isolates obtained from urine samples of patients with UTI treated in three hospitals of Peru in order to generate scientific evidence that will help the competent authorities to make better decisions regarding the rational use of antimicrobials in these patients.

Materials and methods

Study type

Retrospective, cross-sectional, descriptive study. All tests for bacterial identification, antimicrobial susceptibility and molecular detection of beta-lactam resistance genes were performed at the Molecular Biology Research Laboratory of the Professional School of Human Medicine of the Universidad Peruana Unión.

Sample

118 isolates of *E. coli* and *K. pneumoniae* obtained from urine samples of patients with UTI processed between April and August 2019 in the laboratories of the microbiology area of 2 hospitals operated by the Social Security (ESSALUD) and one by the Ministry of Health (MINSA) of Peru were analyzed: 40 isolates from the Hospital de Huaycán (level of care II-1), located in the province of Lima; 44 from the Hospital Nacional Docente Madre Niño San Bartolomé (level of care III-1), located in the province of Lima; and 34 from the Hospital Nacional Alberto Sabogal Sologuren (level of care IV-4), located in the province of Callao.

Bacterial identification and antimicrobial susceptibility

Reactivation of the 118 isolates was performed in tryptic soy broth and then isolated on MacConkey agar.

To identify bacteria and assess antimicrobial susceptibility, the MicroScan automated system (Autoscan-4) was used with panels for gram-negative bacteria (MicroScan@ Neg Combo Panel Type 66) and following the manufacturer's instructions.[8] In each test, 16 antimicrobials were used per isolate and minimum inhibitory concentration values were calculated to identify the antimicrobial susceptibility or resistance of the isolates; the cut-off points used were those recommended by the Clinical and Laboratory Standards Institute 2020.[9] It was established that an isolate was multidrug-resistant when it showed resistance to antimicrobials from 3 or more antimicrobial families.

Finally, bacterial phenotypic identification of ESBL isolates was performed by reading MicroScan Type 66 panels (SMN: 1711680) with the LabPro Command Center software.[8]

Molecular detection of beta-lactam resistance genes

Extraction of bacterial genomic DNA

Bacterial genomic DNA was extracted with the innuPREP Bacteria DNATM kit using the silica column-based DNA extraction method and following the manufacturer's protocol (Analytikjena, Germany).[10] To perform this procedure, it was necessary for the isolates to be in the logarithmic phase of bacterial growth. Once the bacterial genomic DNA was extracted, it was stored at -20°C until the corresponding polymerase chain reaction (PCR) test was performed.

Polymerase chain reaction test

A conventional endpoint PCR was performed to identify each ESBL gene (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$). The primers used were those described by Kiratisin *et al.*[11] and Arce-Gil *et al.*,[12] which are listed in Table 1.

Table 1. Primers used for the detection of extended-spectrum beta-lactamase genes.

Gene	Amplicon (Pb)	Primers	Sequence
bla$_{CTX-M}$	544	CTX-M-F	GAAGGTCATCAAGAAGGTGCG
		CTX-M-R	GCATTGCCACGCTTTTCATAG
bla$_{SHV}$	868	SHV-F	TGGTTATGCGTTATATTCGCC
		SHV-R	GGTTAGCGTTGCCAGTGCT
bla$_{TEM}$	931	TEM-F	TCCGCTCATGAGACAATAACC
		TEM-R	TTGGTCTGACAGTTACCAATGC

F: Forward; R: Reverse; CTX-M: cefotaxime; TEM: temoniera; SHV: sulfhydryl variable; bp: base pairs; ***bla***: beta-lactamase genes.
Source: Own elaboration.

The master mix for the conventional PCR contained: 10µl of 10x PCR buffer including $MgCl^2$ (abm), 1µl of dNTP (abm), 0.5µl of Primer-F (10 uM), 0.5µl of Primer-R (10 uM) (Macrogen, South Korea), 17.3µl of ultrapure water (Invitrogen), 0.2µl of Taq DNA polymerase (0.5U) (abm), and 2.5µl of genomic DNA previously extracted from each bacterial

isolate. This was done according to the indications described in the standardized protocol of the Molecular Biology Research Laboratory of the Professional School of Human Medicine of the Universidad Peruana Unión.

The PCR was performed in a T100TM thermal cycler (Thermal Cycler, Bio-Rad, United States) under the following parameters: 1 cycle of initial denaturation at 94°C for 7 minutes; 35 cycles of denaturation at 94°C for 50 seconds, hybridization at 55°C for 1 minute, and polymerase elongation at 72°C for 60 seconds; and 1 cycle of final polymerase elongation at 72°C for 5 minutes.

Agarose gel electrophoresis

The amplification products obtained with the PCR were visualized by electrophoresis in 1.5% agarose gels (Cleaver Scientific) using TAE 1x buffer (TRIS-acetate-EDTA) and Safe-Green™ visualizing agent (abm). A 100 bp molecular weight marker was used during electrophoresis (opti DNA Marker-abm™). Electrophoresis was performed at 120 volts (v) for 40 minutes in the runSTATION complete documentation electrophoresis system (Cleaver Scientific, United Kingdom) following the standardized protocol for genomic DNA electrophoresis of the Molecular Biology Research Laboratory of the Professional School of Human Medicine at the Universidad Peruana Unión.

Statistical analysis

The data obtained from the isolates analyzed were entered into a database created in Microsoft Excel and analyzed in the R software version 4.1.3 using descriptive statistics (absolute and relative frequencies).

Ethical considerations

The study was approved by the Research and Ethics Committee of the Universidad Peruana Unión as per minutes 2019-CEUPeU-0001 of January 23, 2019, and followed the ethical principles for biomedical research on human subjects established in the Declaration of Helsinki.[13] Furthermore, confidentiality of the participants' data was guaranteed and informed consent was not required because only isolates were analyzed.

Results

Of the 118 isolates analyzed, 105 were identified as *E. coli* and 13 as *K. pneumoniae*. According to the institution, the level II-1 hospital identified 36 isolates of *E. coli* and 4 of *k. pneumoniae*; the level III-1 hospital found 40 of *E. coli* and 4 of *K. pneumoniae;* and the level IV-4 hospital detected 29 of *E. coli* and 5 of *K. pneumoniae*.

All isolates were multidrug resistant, and the majority showed resistance to the antimicrobials aztreonam (75.42%), ceftazidime (72.03%), ciprofloxacin (72.08%), cefotaxime (61.01%), cefepime (50.84%), nitrofurantoin (46. 61%), ampicillin/sulbactam (71.18%), gentamicin (46.61%), trimethoprim/sulfamethoxazole (44.91%), tobramycin (54.23%), amikacin (3. 38%), ertapenem (0.84%), imipenem (10.16%), meropenem (0.84%) tigecycline (2.54%), and piperacillin/tazobactam (4.23%). The resistance profile is described in Table 2.

Table 2. Antimicrobial resistance profile of the 118 isolates tested.

Hospital	Bacterial species	No. of isolates	n (%)														
			AMK	ATM	CAZ	CIP	ETP	CTX	FEP	NIT	SAM	GEN	IPM	MEM	STX	TGC	TOB	TZP
Hospital de Huaycán (level II-1)	*Escherichia coli*	36	0 (0)	10 (27.78)	6 (16.60)	14 (38.80)	0 (0)	6 (16.60)	7 (19.40)	36 (100)	23 (63.80)	12 (33.33)	0 (0)	0 (0)	21 (58.33)	0 (0)	11 (30.50)	0 (0)
	Klebsiella pneumoniae	4	0 (0)	1 (25)	1 (25)	2 (50)	0 (0)	2 (50)	1 (25)	4 (100)	1 (25)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)	1 (25)	0 (0)
Hospital Nacional Alberto Sabogal Sologuren (level IV-4)	*Escherichia coli*	29	0 (0)	29 (100)	29 (100)	26 (89.60)	0 (0)	29 (100)	29 (100)	2 (6.80)	24 (82.70)	15 (51.70)	10 (3.40)	0 (0)	19 (65.50)	0 (0)	18 (62)	0 (0)
	Klebsiella pneumoniae	5	0 (0)	5 (100)	5 (100)	3 (60)	1 (20)	5 (100)	5 (100)	2 (40)	5 (100)	3 (60)	1 (20)	1 (20)	2 (40)	1 (20)	3 (60)	1 (20)
Hospital Nacional Docente Madre Niño San Bartolomé (level III-1)	*Escherichia coli*	40	4 (10)	40 (100)	40 (100)	37 (92.50)	0 (0)	26 (65)	16 (40)	9 (22.50)	28 (70)	23 (57.50)	1 (2.50)	0 (0)	27 (67.50)	1 (2.50)	28 (70)	4 (10)
	Klebsiella pneumoniae	4	0 (0)	4 (100)	4 (100)	4 (100)	0 (0)	4 (100)	2 (50)	4 (100)	3 (75)	2 (50)	0 (0)	0 (0)	3 (75)	1 (25)	3 (75)	0 (0)
Total	*Escherichia coli*	105	3.39	75.23	71.42	73.33	0.95	58.09	49.52	44.76	71.42	47.61	10.47	0.00	63.80	0.95	54.28	3.80
	Klebsiella pneumoniae	13	0.00	76.92	76.92	69.23	0.00	84.61	61.53	76.92	69.23	38.46	7.69	7.69	46.15	15.38	53.84	7.69

AMK: amikacin; ATM: aztreonam; CAZ: ceftazidime; CIP: ciprofloxacin; ETP: ertapenem; CTX: cefotaxime; FEP: cefepime; NIT: nitrofurantoin; SAM: ampicillin with sulbactam; GEN: gentamicin; IPM: imipenem; MEM: meropenem; SXT: trimethoprim sulfamethoxazole; TGC: tigecycline; TOB: tobramycin; TZP: piperacillin with tazobactam; TZP: piperacillin with tazobactam.
Source: Own elaboration.

The study showed that 102 of the 105 *E. coli* isolates and 13 *K. pneumoniae* isolates expressed at least one ESBL gene, with bla_{TEM} being the most frequently expressed (45.76%), followed by *bla*$_{CTX\text{-}M}$ (34.74%), and *bla*$_{SHV}$ (28.81%) (Figure 1). Furthermore, the most frequent ESBL gene in *E. coli* isolates was *bla*$_{CTX\text{-}M}$ in the level III-1 hospital (65%) and *bla*$_{TEM}$ in the level II-1 (50%) and IV-4 hospitals (31.30%). In turn, in *K. pneumoniae* isolates, the most prevalent ESBL genes were *bla*$_{CTX\text{-}M}$ and *bla*$_{SHV}$ in the level III-1 hospital (100% each), and *bla*$_{SHV}$ in the level II-1 (100%) and IV-4 hospitals (100%) (Figure 2).

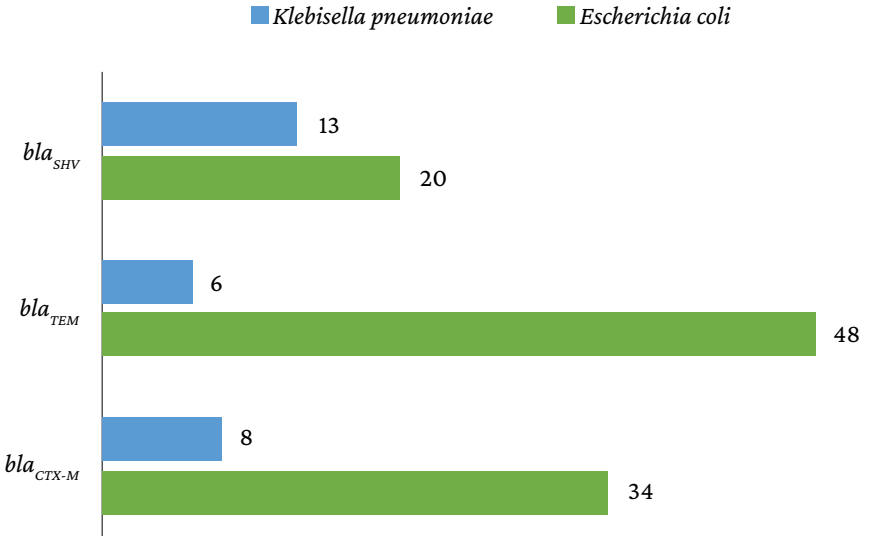

Figure 1. Distribution of genes in total isolates of *Escherichia coli* (n=102) and *Klebsiella pneumoniae* (n=13).
CTX-M: cefotaxime; TEM: temoniera; SHV: sulfhydryl variable; bla: beta-lactamase genes.
Source: Own elaboration.

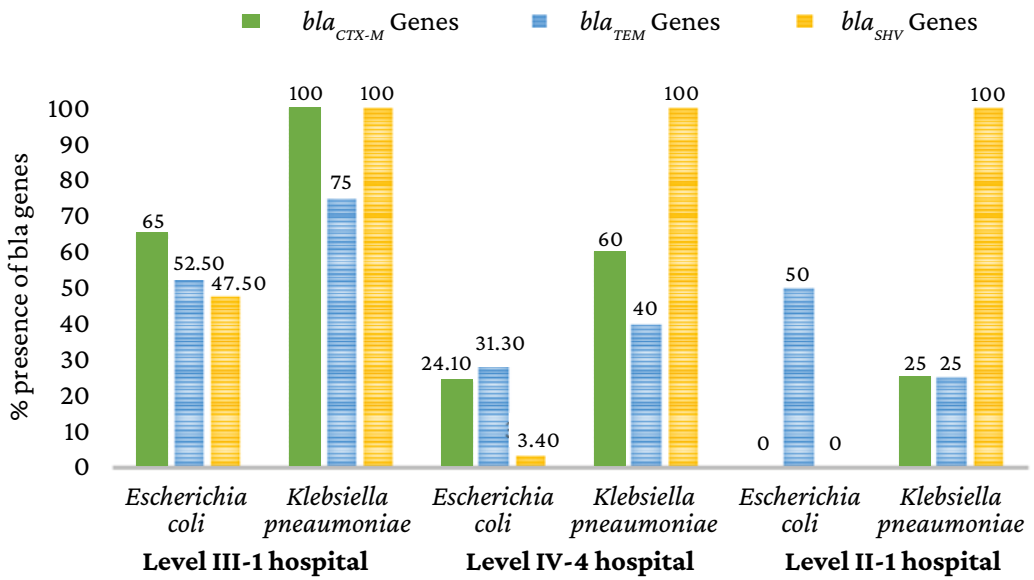

Figure 2. Frequency of beta-lactamase genes in *Escherichia coli* and *Klebsiella pneumoniae* isolates (n=118). CTX-M: cefotaxime; TEM: temoniera; SHV: sulfhydryl variable; bla: beta-lactamase genes.
Source: Own elaboration.

The data obtained showed a high frequency of coexistence of ESBL genes in the *E. coli* isolates (n=30, 28.57%) and in the *K. pneumoniae* isolates (n=8, 61.53%). Moreover, co-expression of 3 genes was observed in 12.71% of all isolates (9.52% of *E. coli* and 38.46% of *K. pneumoniae*) and co-expression of 2 genes in 21.18% (20.95% of *E. coli* and 23.07% of *K. pneumoniae*).

Regarding the distribution of coexistence of ESBL genes by hospital, the findings were as follows:

i) The highest frequency of coexistence in *E. coli* isolates occurred in the level III-1 hospital (56.81%), with coexistence of 3 genes (*blaCTX-M-blaTEM-blaSHV*) being the most frequent (22.50 %), followed by the following coexistences of 2 genes: *blaCTX-M-blaTEM* (20%), *blaCTX-M-blaSHV* (15 %), and *blaTEM-blaSHV* (7.50 %). Meanwhile, in the level IV-4 hospital, the coexistence of 2 ESBL genes was as follows: *blaCTX-M-blaTEM* in 17.20% of the isolates, and *blaTEM-blaSHV* in 3.40%. Finally, in the level II-1 hospital, the *E. coli* isolates did not exhibit coexistence of the ESBL genes studied in the present study.

ii) The highest frequency of coexistence in *K. pneumoniae* isolates occurred in the level III-1 hospital (n=4, 100%), where all 3 genes coexisted in 75% of isolates and 2 genes (*blaCTX-M-blaSHV*) in 25%. Moreover, in the level IV-4 hospital (n=5), the coexistence of the 3 genes was detected in 40% of the isolates and of 2 genes (*blaCTX-M-blaSHV*) in 20%. Finally, in the level II-1 hospital (n=4), the coexistence of only 2 genes (*blaTEM-blaSHV*) was evidenced in 25%.

Discussion

In the present study, all isolates were found to be multidrug resistant. In addition, high rates of resistance to different antibiotics were found, such as aztreonam (75.42%), ceftazidime (72.03%), ciprofloxacin (72.08%), ampicillin/sulbactam (71. 18%), cefotaxime (61.01%), tobramycin (54.23%), cefepime (50.84%), nitrofurantoin (46.61%), gentamicin (46.61%), and trimethoprim/sulfamethoxazole (44.91%). These percentages are higher than those found in 2017 by Miranda-Estrada *et al.*,[14] who analyzed 107 *E. coli* isolates, 50 obtained from a locality in central Mexico and 57 from a locality in southwestern Mexico,

and reported that the rates of resistance to ceftazidime, ciprofloxacin, cefotaxime, tobramycin, cefepime, nitrofurantoin, and gentamicin were 57%, 45.8%, 57%, 28%, 15.9%, 13.1%, and 32.9%, respectively. These differences show an increase in antibiotic resistance in recent years and lead us to believe that over time it could reach 100% if corrective public health measures are not taken.

Similarly, a major difference was evident with the findings reported by Lopez-Banda *et al.*,[15] who studied 108 *E. Coli* isolates obtained between 2008 and 2010 from urine samples of Mexican women with UTI and reported that resistance rates to aztreonam, ceftazidime, cefotaxime, and cefepime were 0%, whereas in the present study resistance rates to these antibiotics were above 50%. Also, the resistance rates reported by López-Banda *et al.*[15] for ciprofloxacin (62.3%), gentamicin (27.8%), tobramycin (43.5%), imipenen (1.9%), meropenen (0%) were lower than those found in the present study.

On the other hand, in the present study *bla*$_{TEM}$ was the most frequently expressed ESBL gene in the isolates analyzed (45.76%), followed by *bla*$_{CTX\text{-}M}$ (34.74%) and *bla*$_{SHV}$ (28.81%), which differs from the findings reported by Galván *et al.*,[16] who found a higher prevalence of the *bla*$_{CTX\text{-}M}$ gene (79.20%), followed by *bla*$_{TEM}$ (37.7%) and *bla*$_{SHV}$ (5.7%) in a study of 53 ESBL-producing *E. coli* isolates obtained from urine cultures performed between September and December 2012 in a laboratory in Lima. This shows an increase in the frequency of isolates with the *bla*$_{TEM}$ gene and a reduction in isolates with the *bla*$_{CTX\text{-}M}$ gene.

In the same way, Mirkalantari *et al.*[2] performed between 2014 and 2016 a study in which they included 183 *E. coli* isolates obtained from urine samples of patients with community-acquired UTI, in which they showed that the most frequent ESBL gene was *bla*$_{CTX\text{-}M}$ (69.5%), followed by *bla*$_{TEM}$ (47.4%), and *bla*$_{SHV}$ (44%), which differs from the present study where the gene with the highest incidence was *bla*$_{TEM}$; furthermore, in this study, coexistence of 3 and 2 genes was only recorded in 25.4% and 32.2% of the isolates, respectively. Likewise, Gonzales-Rodriguez *et al.*[17] reported that in 35 ESBL-producing *E. coli* isolates obtained in 2018 from urine samples of older adults residing in geriatric homes in metropolitan Lima the *bla*$_{CTX\text{-}M}$ gene was the most frequent (57.1%), followed by *bla*$_{TEM}$ (54.3%) and *bla*$_{SHV}$ (51.4%), which also differs with what was found in the present study.

In the present study, the coexistence of 3 genes was observed in 12.71% of the isolates (9.52% of *E. coli* and 38.46% of *K. pneumoniae*), a figure higher than that reported by Luna *et al.*,[18] who found the coexistence of these 3 genes in 4.9% of *E. coli* isolates in a study of 61 urine culture isolates (49 of *E. coli*) obtained from urinary samples of adult patients treated between 2017 and 2018 in the Peruvian jungle. On the other hand, the coexistence of 2 genes was observed in 21.18% of the isolates (20.95% in *E. coli* and 23.07% in *K. pneumoniae*), with this figure being similar to that described by Galván *et al.*[16] in a study in which they evaluated 53 ESBL-producing *E. coli* isolates and where coexistence of *bla*$_{CTX\text{-}M}$ and *bla*$_{TEM}$ was observed in 24% of the isolates.

Regarding the strengths of the present study, it should be noted that the data reported here allow us to know the rate of antibiotic resistance in urine samples of patients with UTI treated in hospitals in Lima and Callao using molecular biology techniques, which are more sensitive and accurate than a phenotypic study. On the other hand, as a limitation, although there was interest in analyzing samples from more hospitals in Lima to obtain more data and a better view of antibiotic resistance in hospitals with a large number of patients, this was not possible due to administrative barriers unrelated to the study; therefore, further studies should, if possible, include isolates from more hospitals in the region to confirm these data.

Conclusion

In the present study, all isolates tested were multidrug resistant to antimicrobials. Moreover, coexistence of ESBL genes was observed in almost one third of all isolates, which shows that antibiotic resistance is a real problem in public hospitals in Lima and Callao. Considering the above, this study provides data that can contribute to offering better antimicrobial treatment to patients with urinary tract infections and to gaining insight into the major problem of antimicrobial resistance in public hospitals.

Conflicts of interest

None stated by the authors.

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