BRIEF REPORT

FREQUENCY OF COLISTIN RESISTANCE IN *Pseudomonas aeruginosa:* FIRST REPORT FROM PERU

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This study is part of the bachelor's thesis of Delsy Barrantes Salinas and Marjhory Zarate Estrada, "Resistencia antimicrobiana a colistina en Pseudomonas aeruginosa aisladas de tres establecimientos de salud de Lima, Perú", Facultad de Medicina Honorio Delgado; Universidad Peruana Cayetano Heredia, presented in 2020.

ABSTRACT

This study aimed to determine the frequency of colistin resistance in *Pseudomonas aeruginosa* isolates obtained from three healthcare facilities in Lima and cryopreserved at the Laboratorio de Resistencia Antimicrobianos e Inmunopatología of the Universidad Peruana Cayetano Heredia (UPCH). The colistin broth disk elution method was used for the phenotypic identification of colistin resistance. We detected the expression of the *mcr-1* gene by using the phenotypic diffusion method with combined colistin and ethylenediaminetetraacetic acid (EDTA) disks; and polymerase chain reaction (PCR) was used for molecular identification of the gene. Of the 97 isolates, 7 (7.2%) were resistant to colistin; however, none carried the *mcr-1* gene. This is the first report from Peru on clinical isolates of colistin-resistant *Pseudomonas aeruginosa*, which suggests the need for implementation of appropriate methodologies for the epidemiological surveillance of colistin-resistant pathogens.

Keywords: *Pseudomonas aeruginosa*; Antimicrobial Drug Resistance; Colistin; Peru (Source: MeSH NLM).

INTRODUCTION

Pseudomona aeruginosa is an opportunistic pathogen of clinical and epidemiological relevance, associated with nosocomial infections including sepsis, pneumonia and urinary tract infections. Due to the intrinsic, acquired and adaptive resistance mechanisms that characterize this pathogen, the therapeutic alternatives for the infections it causes are limited to only a few groups of antibiotics. However, the high selective pressure exerted by antibiotics in hospital environments has generated a rapid global spread and dissemination of multidrug-resistant (MDR) *P. aeruginosa* clones [(1)].

The concern related to this bacterium has been highlighted by the World Health Organization (WHO) and in February 2017 included carbapenem-resistant *P. aeruginosa* in the list of pathogens with critical priority, for which new antibiotics are required and that has forced the adoption of more aggressive treatment strategies, for example, the use of older antibiotics such as colistin, despite its high toxicity [(2)].

Colistin is a cyclic polypeptide antibiotic of cationic lipid nature discovered in 1947. It acts in the lipopolysaccharides (LPS) of the outer membrane of gram-negative bacilli (GNB), bin-

Cite as: Zarate M, Barrantes D, Cuicapuza D, Velasquez J, Fernández N, Salvatierra G, *et al.* [Frequency of colistin resistance in *Pseudomonas aeruginosa*: first report from Peru]. Rev Peru Med Exp Salud Publica. 2021;38(2):308-12. doi: https://doi.org/10.17843/rpmesp.2021.382.6977.

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Received: 12/29/2020
Approved: 04/07/2021
Online: 05/26/2021

https://doi.org/10.17843/rpmesp.2021.382.6977

ding electrostatically to lipid A, displacing Mg2+ and Ca2+ cations and inducing the opening of the outer membrane, osmotic changes in the periplasm and subsequent bacterial lysis [3]. In clinical practice, adverse effects such as nephrotoxicity and neurotoxicity limited its use for years; however, colistimethate sodium is currently used as an inactive prodrug, which, in an aqueous medium, is transformed into colistin, thus reducing its harmful effects [4].

Different mechanisms of resistance to this polymyxin have been identified; one of them is the result of modifications of the lipopolysaccharides of the outer membrane (in lipid A) mediated by the addition of a 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (PEtN). This modification of lipid A is regulated by the two-component systems (TCS) PhoP-PhoQ (PhoPQ) and PmrA-PmrB (PmrAB), resulting in the reduction of the outer membrane negative charge that generates colistin resistance [1,5].

On the other hand, plasmid resistance to colistin called *mcr-1* (mobile colistin resistance), a member of the family of phosphoethanolamine transferase enzymes, capable of modifying the target site of colistin, decreasing its affinity for lipid A by adding phosphoethanolamine, was described for the first time in China in 2015 [6]. That same year, in Vietnam, the first clinical case was reported in a diarrheal sample, cryopreserved until 2008, in which a *Shigella sonnei* carrying the gene was isolated [7]. To date, 10 genes homologous to *mcr-1* and 21 allelic variants have been characterized [8]. This gene is transmitted by plasmids, mainly associated with the X4 incompatibility group (IncX4), which allows its horizontal transfer between different GNB species [9].

In 2016, Arcilla *et al*. reported two ESBL-producing *Escherichia coli* strains carrying the *mcr-1* gene isolated from diarrheal samples of Dutch travelers from Peru, Colombia and Bolivia after their return to the Netherlands [10]. In Latin America, the *mcr-1* gene has been reported in Argentina, Venezuela, Colombia, Ecuador, and Brazil [11]. In Peru, in 2018, Ugarte *et al*. reported for the first time isolates of *E. coli* and *Klebsiella pneumoniae* carrying *mcr-1* [12]. The plasmid mechanism mediated by the *mcr-1* gene was first detected in *P. aeruginosa* and *Acinetobacter baumannii* in a multicenter study conducted in Pakistan in 2019 [13]. In Peru, colistin resistance has not been reported so far, nor has the presence of the *mcr-1* gene in *P. aeruginosa* isolates of clinical origin.

The emergence of colistin resistance through horizontal transfer of conjugative plasmids is of increasing concern worldwide due to its rapid dissemination.

KEY MESSAGES

Motivation for conducting the study: Given the possibility of underreporting of colistin resistance in hospitals and clinics in Peru, three high-performance methodologies were used to determine the frequency of colistin resistance by chromosomal and plasmid mechanisms.

Main findings: We identified 7.2% of colistin-resistant *P. aeruginosa* isolates not carrying the *mcr-1* gene.

Implications: Colistin resistance was determined for the first time in *Pseudomona aeruginosa* in Peru.

Based on the above, this study was carried out to determine the frequency of colistin resistance by the colistin broth disk elution method (CBDE), the frequency of *mcr-1* gene expression by the combined colistin-EDTA disk diffusion test (CDT) and the presence of the *mcr-1* gene by polymerase chain reaction (PCR) in *P. aeruginosa* isolates from three health facilities in Lima, Peru.

THE STUDY

A descriptive cross-sectional study was carried out in which *P. aeruginosa* isolates of clinical origin from three health facilities in Lima (two public and one private) were evaluated from January 2018 to October 2019. The isolates were cryopreserved in the strain bank of the Antimicrobial Resistance and Immunopathology Laboratory of the Research and Development Laboratories (LID) of the Universidad Peruana Cayetano Heredia (UPCH).

Phenotypic detection of colistin resistance

A total of 97 isolates of *P. aeruginosa* were recovered in Cetrimide selective medium (Liofilchem, Italy) and incubated for 24 hours at 35 °C. Colistin resistance was determined using the CBDE method, described by the Antimicrobial Referral Laboratory of Argentina "Dr. Carlos G. Malbrán" [14]. The isolates were classified, based on the results of the minimum inhibitory concentration (MIC), intermediate (MIC ≤ 2 µg/mL) or resistant (MIC ≥ 4 µg/mL), for which we used the cut-off points established in the M100-ED30: 2020 guide of the Clinical and Laboratory Standard Institute (CLSI) [15].

Combined disc diffusion test

Isolates were inoculated onto Müller Hinton agar (Merck, Germany) from a tube with physiological saline at a concentration equivalent to tube no. 0.5 on the McFarland scale. Two sensitivity discs were used, one with 10 µg of colistin (Oxoid, England) and the second with 10 µg of colistin plus 10 µL of EDTA (Sigma-Aldrich, USA) at a concentration of 100 mM. A difference ≥ 3 mm between the inhibition halos of colistin plus EDTA compared to that observed with the colistin disc alone was considered positive for *mcr-1* gene expression; the method was standardized by Esposito *et al.* [16].

Detection of the *mcr-1* gene

For molecular identification of the gene, genomic DNA was extracted from the 97 isolates by the heat shock method; and PCR for the *mcr-1* gene was carried out using the following primers: forward: CLR5-F1 (5'-CGGTCAGTCCGTTTGTTC-3') and reverse: MCR-indel-R1 (5'-CTTGGTCGGTCGGTCTGTAGGG-3). Amplification was carried out under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec; 45 °C for 30 sec; 72 °C for 30 sec; and final extension at 72 °C for 10 min, according to the methodology described by Liu YY [6]. DNA size was verified with DNA ladder 100 bp (Thermo Scientific, USA), considering products of 309 bp.

For validation of the results, in all tests we used an *E. coli* strain molecularly characterized as a carrier of the *mcr-1* gene as a positive control, from the Laboratory of Antimicrobial Resistance and Immunopathology, and an *E. coli* ATCC 25922 was used as a negative control.

Data analysis

The statistical program STATA, version 16.0 (StataCorp, College Station, TX, USA) was used to report absolute frequencies and percentage of the variables of interest obtained from the study.

Ethical Aspects

The protocol was registered and approved in the Decentralized System of Information and Monitoring of Research (SIDISI:103444) - University Directorate of Research, Science and Technology (DUICT); and, prior to its execution, the UPCH Ethics Committee (CIE-UPCH: CAREGOR-VEI-141-19) evaluated it. The study followed the guidelines of good practices and ethics in biomedical research.

FINDINGS

Of the total of 97 *P. aeruginosa* isolates of clinical origin, the CBDE method identified 7 (7.2%) colistin-resistant isolates with an MIC equal to 4 µg/mL; 49 (50.5%) had an MIC equal to 2 µg/mL; and 41 isolates (42.3%) had an MIC less than or equal to 1 µg/mL and were categorized as intermediate to colistin. The antibiotic concentration that inhibited 50% and 90% of the isolates (MIC_{50} and MIC_{90}) was 2 µg/mL. Colistin-resistant isolates were obtained from different clinical samples, such as wound secretion (n = 2), blood (n = 3), urine (n = 1) and feces (n = 1). Of the total colistin-resistant isolates, 2 were from health facility 1 (6.9%, n = 29), 2 from health facility 2 (12.5%, n = 16) and 3 from health facility 3 (5.8%, n = 52) (Table 1). The result of the CDT method was negative for all strains evaluated. Likewise, the PCR test did not detect any isolates carrying the *mcr-1* gene (Table 2).

DISCUSSION

The results of this study show that 7.2% of *P. aeruginosa* isolates were resistant to colistin. This finding is similar to the 5.2% reported for Latin America [17] and Europe 4% [3]. In Peru, colistin resistance had not been reported in *P. aeruginosa* prior to this study [18].

Table 1. Bacterial resistance to colistin and characteristics of clinical isolates.

Characteristic	Total (N = 97)	Colistin [a]	
		Resistant (n = 7)	Intermediate (n = 90)
	N (%)	n (%)	n (%)
Institution			
Health center 1	29 (29.9)	2 (6.9)	27 (93.1)
Health center 2	16 (16.5)	2 (12.5)	14 (87.5)
Health center 3	52 (53.6)	3 (5.8)	49 (94.2)
Sample type			
Bronchial aspirate	4 (4.1)	-	4 (100.0)
Bile	1 (1.0)	-	1 (100.0)
Stool culture	2 (2.1)	1 (50.0)	1 (50.0)
Thoracic drainage	1 (1.0)	-	1 (100.0)
Sputum	1 (1.0)	-	1 (100.0)
Blood culture	11 (11.3)	3 (27.3)	8 (72.7)
Secretions	59 (60.8)	2 (3.4)	57 (96.6)
Urine culture	18 (18.6)	1 (5.6)	17 (94.4)

[a] Colistin broth disk elution method (CBDE)

https://doi.org/10.17843/rpmesp.2021.382.6977

Table 2. Colistin broth disk elution method, combined disk diffusion test and polymerase chain reaction for *mcr-1* in *Pseudomonas aeruginosa* colistin-resistant phenotype.

Strain code	Health center	Sample type	CBDE	CDT	PCR *mcr-1* gene
2041	1	Secretion culture	4 mg/mL (R)	0 mm	Negative
2043	1	Urine culture	4 mg/mL (R)	0 mm	Negative
1002	2	Stool culture	4 mg/mL (R)	0 mm	Negative
1005	2	Blood culture	4 mg/mL (R)	2 mm	Negative
430	3	Secretion culture	4 mg/mL (R)	1 mm	Negative
631	3	Blood culture	4 mg/mL (R)	1 mm	Negative
806	3	Blood culture	4 mg/mL (R)	1 mm	Negative

CBDE: colistin broth disk elution method; CDT: combined disk diffusion test; PCR: polymerase chain reaction; R: resistant.

Several studies have determined that conventional methods used in antibiograms, such as agar disc diffusion, Epsilon test, even commercial systems such as VITEK (Biomerieux), Phoenix (Becton Dickinson), used in hospitals throughout the country, generate false susceptibility to colistin, which could be leading to underreporting of real resistance [19].

Given the current importance of colistin and the technical problems related to the determination of resistance, a study with methods of proven effectiveness was necessary. The CBDE test is a good alternative in terms of cost-effectiveness, efficacy and performance, and can be applied routinely in clinical microbiology laboratories [3,19]. The study conducted by Humphries, *et al.* supports the application of this test in *P. aeruginosa* with an essential concordance of 94.4% and a categorical concordance of 97.9% with the MICs obtained by the broth microdilution method considered as the reference test. Based on these results, the CLSI Subcommittee on Antimicrobial Susceptibility Testing (AST) approved the CBDE method for resistance testing in Enterobacteriaceae and P. *aeruginosa* [18, 20].

The use of chelators such as EDTA for the inhibition of phosphoethanolamine transferase produced by the *mcr-1* gene has become widespread in clinical microbiology laboratories. The CDT test for presumptive detection of phenotypic expression of *mcr* gene variants by alteration of the zeta potential showed promising results, as it is a rapid and inexpensive alternative in *E. coli* isolates [16]. Our results showed that *P. aeruginosa* isolates with a colistin-resistant phenotype (MIC ≥4 µg/mL) did not have a difference ≥ 3 mm in the diameter of the inhibition zone of the colistin/EDTA disk compared with the colistin disk, a feature that is related to the absence of the *mcr-1* gene. These results are similar to those published by Abd El-Baky, *et al.* who found that 50.0% of *P. aeruginosa* with an MIC ≥ 32 µg/mL and negative CDT did not have the *mcr-1* gene [5].

In this study, the screening of the *mrc-1* gene by PCR was negative for all the strains evaluated, which indicates that the resistance to colistin that we have detected could be due to the presence of allelic variants of the gene or to chromosomal mechanisms that we have not been able to identify with the methodology used. It is important to consider that the molecular identification of the *mcr-1* gene and its variants does not necessarily confirm colistin resistance in bacteria that carry it, since susceptible phenotypes may be present [8].

Recently, it was identified that the main mechanism responsible for chromosomal colistin resistance in GNB is the result of specific mutations, environmental stimuli and inactivation by insertion into the PhoP-PhoQ (PhoPQ) and PmrA-PmrB (PmrAB) two-component system (TCS), leading to overexpression of the pmrHFIJKLM operon. This results in the addition of L-Ara4Ns and PEtNs to the lipid A of the outer membrane of *P. aeruginosa*, which decreases the net negative charge of phosphate residues that affects colistin affinity [4,5].

Despite the alarming emergence of colistin-resistant variants of *P. aeruginosa*, the implementation of sensitive and specific susceptibility testing in the routine work of clinical microbiology laboratories has been slow. In this study, we used well-defined protocols combining phenotypic susceptibility testing and molecular methods. However, there are important limitations in our research; the isolates included in the study were obtained from three health facilities during

a specific period of time, therefore, our results cannot be inferred to the current situation in the hospital setting; likewise, genes homologous to *mcr-1* were not evaluated; and the chromosomal mechanisms involved in colistin resistance were not characterized.

In conclusion, the CBDE method identified 7.2% of clinical isolates of *P. aeruginosa* resistant to colistin, which is the first report of resistance to this antibiotic in Peru. This finding shows the need to implement, in our country, surveillance of colistin resistance in *P. aeruginosa* through the application of appropriate methodologies.

Authorship contributions: All authors participated in the research idea, conception of the article, collection of data and study material, and writing of the article. All authors approved the final version of the manuscript.

Funding: The study was funded with resources from the Laboratory of Antimicrobial Resistance-LID-UPCH.

Conflicts of interest: The authors have no conflicts of interest to declare.

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 https://doi.org/10.17843/rpmesp.2021.382.6977