BRIEF REPORT

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Colistin resistance screening by 3 µg/ml colistin agar in Carbapenemase-producing *Enterobacterales*

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Abstract

Background: In low- and middle-income countries, the use of colistin in therapeutic regimens is common, to treat infections produced for Carbapenemase-producing *Enterobacterales* (CPE) due to limited access to the recently discovered-approved antibiotics. Furthermore, the technical limitations to perform colistin susceptibility tests make it difficult to assess the suitability of this treatment for each patient, as well as to monitor the rates of resistance. In the present study, we describe the use of agar dilution using a unique colistin concentration of 3 µg/ml to discriminate isolates with colistin resistance in CPE obtained from clinical samples.

Methods: Clinical Laboratory Standards Institute (CLSI) colistin broth microdilution method and dilution agar with a colistin concentration of 3 µg/ml were performed in 168 isolates of CPE obtained from clinical samples in Guayaquil, Ecuador. Broth microdilution was considered our gold standard using CLSI breakpoints as reference (≤2 µg/ml intermediate and ≥4 µg/ml resistant). Categorical agreement was defined as obtaining a reading within the same category with both methodologies.

Results: Isolates obtained from respiratory samples were the most prevalent (26.19%; $n = 44$). *Klebsiella pneumoniae* was the predominant specie (94.04%; $n = 158$). KPC-like carbapenemase was present in all the isolates, and interestingly, colistin resistance was not mediated by MCR-1 production. Categorical agreement between both methods resulted in 97.02%.

Conclusion: We propose the use of dilution agar with a colistin concentration of 3 µg/ml, as a valid method for screening colistin resistance in low- and middle-income countries to monitor resistance and to perform epidemiological studies.

KEYWORDS
broth microdilution, Carbapenemase-producing *Enterobacterales*, colistin dilution agar, colistin resistance, surveillance

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1 | INTRODUCTION

Carbapenemase-producing *Enterobacterales* (CPE) are one of our greatest threats worldwide, and the current increasing incidence correlates with a rise in the mortality rate due to the scarce therapeutic options available.[1,2]

Since the identification of the first carbapenemase KPC-like (*Klebsiella pneumoniae* carbapenemase) in 1996 in the United States,[3] the outlook has been unfortunate. The rapid spread worldwide correlates with the expansion of predominant clonal complexes and the development of new genetic variants, as well as the appearance of other carbapenemases such as New Delhi metallo-beta lactamase (NDM) or the oxacillinases (OXA), mainly OXA-48.[2] Altogether, creates an imperative need toward the use of old antibiotics like colistin as well as the development of new antimicrobials.[4,5]

Even though the recently approved antibiotics such as ceftazidime/avibactam constitute the first line of treatment against CPE, some countries have limited availability. Moreover, combined therapeutic regimens including colistin are broadly used.[6,7]

The evolving resistance to colistin frightens especially certain regions. After the first description of the plasmid encoded *mcr* (mobile colistin resistance) gene in 2015,[8] nine variants have been described in humans, environment, and food samples.[9]

Technical difficulties to assess susceptibility to colistin are one of the greatest limitations for diagnostic as well as epidemiological purposes. The size of the colistin molecule does not allow for its use with the classical disc diffusion method nor diffusion by concentration gradient. Moreover, automated methods are not recommended due to the great variability and error when it comes to assess susceptibility of this particular antibiotic.[10,11] In 2020, the Clinical Laboratory Standard Institute (CLSI) approved two methods to perform colistin resistance (CR) surveillance tests, broth disk elution and colistin agar test based in modified agar dilution method, which consist in the use of serial dilutions between 1 and 4 µg/ml, and finally, colistin broth microdilution (BMD), which is still considered the reference method.[12] These methodologies require specific reagents including the agar media containing different colistin concentrations, posing difficulties to implement these tests as routine techniques in every microbiological laboratory. Unlike the CLSI, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) contemplates the broth microdilution as the only acceptable method for the determination of colistin susceptibility (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_11.0_Breakpoint_Tables.pdf) posing even more hurdles in the detection and diagnostic of this resistance mechanism.

Some of the available commercial methodologies for the detection of CR are chromogenic media such as CHROMagar COL-APSE, or commercial kits such as ComASP colistin, Colistin MAC test, ColiSpot, rapid polymyxin NP test (RPNP), MICRONAUT-MIC Strip, UMIC System and Sensitest™ Colistin, which have proven excellent correlation with the gold standard method.[13] However, these methodologies are not considered as an alternative in low-income laboratories due to their cost.

Based on the available information, we proposed to evaluate if the agar dilution with a colistin concentration of 3 µg/ml allows to differentiate isolates with colistin resistance in CPE obtained from clinical samples.

2 | METHODS AND MATERIALS

2.1 | Bacterial isolates

One hundred and sixty-eight isolates of *Enterobacterales* with carbapenem resistance were included in this study. These isolates were obtained from clinical samples of secondary and tertiary hospitals units from Guayaquil, Ecuador, during between December 2019 and May 2020. One isolate per patient was utilized, if a patient had more than one, we selected an isolate obtained from a sterile site or the isolate with greater resistance. All the isolates included in the study were *bla*KPC positive and *mcr*-1 gene negative.

The isolates were collected by microbiology laboratories in each hospital and sent to us in Stuart medium for further processing in a reference laboratory. Samples were cultured in agar MacConkey (Becton Dickinson) at 37°C for 16–18 h to determine viability as well as purity. Next, they were cultured in CHROMagar SuperCarba (CHROMagar) to confirm the resistance to carbapenems. Bacterial identification was performed using the Vitek 2 Compact System (BioMérieux) and/or conventional biochemical assays. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *K. pneumoniae* BAA ATCC 1705 were used for quality control for bacterial identification tests and chromogenic agar.

Carbapenemase type was molecularly confirmed with a multiplex polymerase chain reaction (PCR) to detect the presence of *bla*KPC, *bla*OXA-48, *bla*VIM, *bla*IMP, and *bla*NDM,[14] and MCR-1 production was detected with a multiplex PCR previously described.[15]

2.2 | Antimicrobial colistin susceptibility

2.2.1 | Broth microdilution method

The minimal inhibitory concentration (MIC) of colistin was determined using broth microdilution, following the recommendations of the CLSI document M07-A8.[16] We used a concentration range between 0.5 and 8 µg/ml Colistin sulfate analytical grade (Sigma-Aldrich. Code C2700000.Batch 3.0) and Mueller Hinton broth with cation adjustment (Thermo Fisher Scientific).

2.2.2 | Agar dilution with colistin 3 µg/ml (CA-3)

The protocol from the CLSI M07-A8 document was followed. A single concentration of 3 µg/ml of colistin sulfate (Sigma-Aldrich. Code C2700000.Batch 3.0) was added to Mueller-Hinton Agar (Becton Dickinson). The agar was prepared and stored at 4°C and it was used

within the next 48 h. The bacterial inoculum was adjusted to 0.5 McFarland Scale using the DensiChek Equipment (BioMérieux Inc). A 1/10 dilution was done and 10 μl of each bacterial inoculate was cultivated. Ten samples per petri dish were placed and incubation was done for 16–18 h at 35°C. The growth of 1 or more colonies in the agar was considered positive with a MIC >3 μg/ml. *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* AR Bank #0349 were used for quality control. We also included three clinical isolates of *E. coli* extended spectrum *Beta-lactamase* (ESBL) with *mcr-1* positive (two obtained from urinary samples and one from blood: MICs ≥8 μg/ml).

2.3 | Data analysis

The broth microdilution was considered as the reference method. The CLSI breakpoints were used (≤2 μg/ml intermediate and ≥4 μg/ml resistant). The categorical agreement (CA) was defined as the percent of the isolates classified within the same susceptibility range by BMD (gold standard) and the method under evaluation. In the case of conflicting results, the results obtained by microdilution were the ones to delineate the category. Very major errors (VME) and major errors (ME) were not identified, because a susceptible category is not defined by CLSI. Minor errors were defined when the BMD identified a resistance isolate and CA-3 shows an intermediate result.

3 | RESULTS

Isolates obtained from respiratory samples were the most prevalent, with 26.19% (*n* = 44) followed by urine and blood samples 23.80% (*n* = 40) and 20.23% (*n* = 34), respectively. The rest of the processed samples were from catheters (8.33%; *n* = 14), surgical wound discharges, (6.54%; *n* = 11), rectal swabs (4.76%; *n* = 8), and others

(7.73%; *n* = 13). Four bacterial isolates were of an unknown origin (2.38%).

K. pneumoniae was the most frequently isolated microorganism, with 94.04% (*n* = 158), followed by *Klebsiella aerogenes* (2.38%, *n* = 4), *Enterobacter cloacae*, and *E. coli*, both isolated in equal percentage (1.79%, *n* = 3).

Fifty-six colistin resistance (33.30%) and 112 (66.66%) colistin intermediate isolates were processed (*n* = 168). Our results demonstrated a categorical agreement of 97.02% (*n* = 163). Five *K. pneumoniae* isolates with MICs 4 μg/ml provided negative results in CA-3 (mE 2.97%).

MICs distribution of all isolates are detailed in Figure 1, and interpretation results for each microorganism are detailed in Table 1.

In addition, 78.57% (*n* = 44) of the colistin resistant isolates presented confluent growth in agar CA-3. In 12 isolates, the results had to be confirmed due to the insufficient growth (<3 colonies), requiring additional work. Importantly, 100% of these microorganisms that needed to be further evaluated, presented colistin resistance demonstrating the robustness of the method studied.

4 | DISCUSSION

The use of colistin as treatment is highly limited by the rapid increase of CPE being isolated in different countries.[4] This rapid increase in resistance creates an imperative need to implement susceptibility tests to further evaluate the suitability of this antibiotic; however, these susceptibility tests have rendered many limitations. Because of this, we evaluated the Agar dilution test described in the CLSI using one single colistin concentration (3 μg/ml).

Our results demonstrated a robust categorical agreement (97.02%), between the use of CA-3 and broth microdilution, and colistin resistant isolates were clearly separated. Even though there is no susceptibility test that uses agar dilution with a single
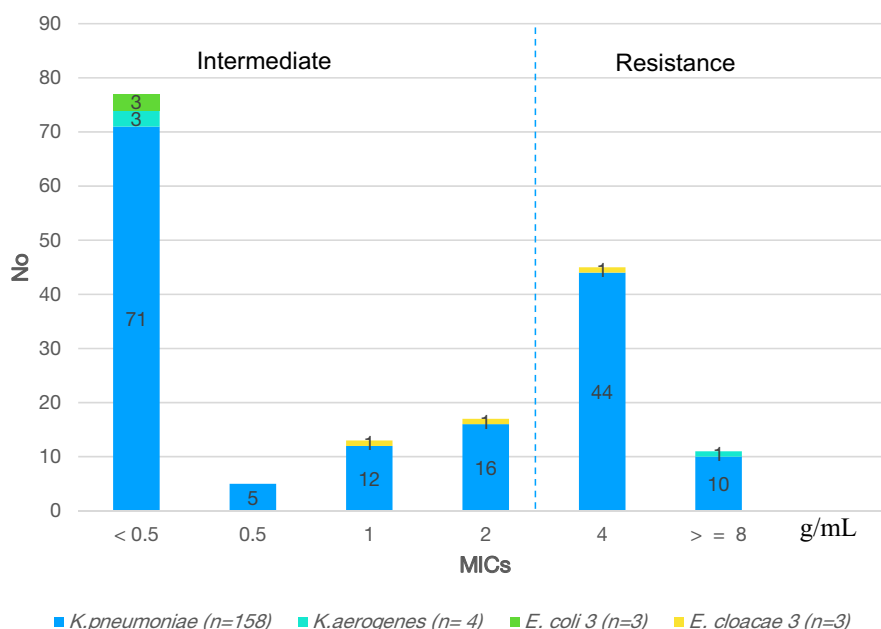

FIGURE 1 Colistin MICs distribution and interpretation according to CLSI breakpoints.

TABLE 1 Comparison of colistin MICs and colistin agar dilution-3 µg/ml results for Carbapenemase-producing *Enterobacterales*

Microorganism	Colistin agar dilution-3 µg/ml Result	MIC distribution[a] (µg/ml) <0.5	0.5	1	2	4	≥8	Interpretation results
Klebsiella aerogenes (n = 4)	Negative	3						CA: 100% mE: 0%
	Positive						1	
Enterobacter cloacae (n = 3)	Negative			1				CA: 100% mE: 0%
	Positive				1	1		
Escherichia coli (n = 3)	Negative	3						CA: 100% mE: 0%
	Positive							
Klebsiella pneumoniae (n = 158)	Negative	71	5	12	16	5*		CA: 96.83% mE: 3.16%
	Positive					39	10	
Total		77	5	13	17	45	11	CA: 97.02% (163) mE: 2.97% (5)

Note: Black line represents the CLSI colistin breakpoint. Minor errors are indicated by *.

Abbreviations: CA, categorical agreement; mE, minor error; MIC, minimal inhibitory concentration.

[a]Results obtained by broth microdilution method.

concentration, our results are similar to those reported by other authors, in regards to the categorical agreement, that included a MIC between 1 and 4 µg/ml.[17] Moreover, the many limitations encountered to test colistin susceptibility make this method a great option to be broadly used in clinical microbiology laboratories. Providing a great alternative for those places where there is no other option.

In 2021, Pasterán et al. described the technique known as Colistin Agar Spot (CAS), based on agar dilution using a single colistin sulfate concentration (3 µg/ml) but with a simplified inoculation (20-mm drop-spot of a 0.5 McFarland bacterial suspension). They established a CA of 98.5% when compared to broth microdilution for *Enterobacterales*.[18] CAS has been used in the microbiology laboratory system in Argentina since 2018 with excellent results. Giacoboni et al, and Yauri-Condor et al, in Peru, reported similar results with CAS for *Enterobacterales*.[19,20] The use of this test has been extended to *P. aeruginosa* and *Acinetobacter baumannii* to identify plasmid-mediated resistance to colistin using CAS with EDTA.[21,22]

Five isolates of *K. pneumoniae* with MIC 4 µg/ml gave negative results in CA-3 (2.97% mE), which could be explained due a large number of isolates with MIC 4 µg/ml (n = 45) was included, and this type of errors are frequently reported to occurred near the breakpoint. Errors in MIC values of 2 µg/ml were not observed, but only 17 isolates were included.[23] Also, the well-known heteroresistance phenomenon, mainly described in *Enterobacter* spp., could be presented in these isolates making it difficult to confront MICs interpretations because it likes to produce different MICs results.[24,25] This phenomenon has been well described in *Enterobacter* spp.; however, we observed it in *K. pneumoniae*, similar to those described by Fóldes et al.[26] Additionally, the possibility of some technical mistakes associated with pipetting cannot be excluded, due to the fact that our study was performed under a routinary laboratory workflow.

Despite this, mE is below 3%, a value recommended by CLSI for susceptibility tests evaluation.[27]

The use of a single concentration facilitates sample-processing allowing to save time and resources while gaining in the accuracy of the diagnostic. To our knowledge, using a single concentration breakpoint to determine colistin resistance is not further studied with agar dilution. Pasterán et al. uses a concentration of 2 µg/ml with poor results (CA: 95.9% and ME: 8.3%) for *Enterobacterales*. Breakpoint concentrations to discriminate colistin-resistance isolates have been mainly used in rapid colorimetric tests. Values of 3.7 µg/ml (Rapid Colistin Disk Elution, CA 98.3%, VME 5.4%),[28] 3.8 µg/ml (Modified Andrade Screening Antimicrobial Test. ASAT. Sensitivity 90.7%, specificity 100%),[23] and 5 µg/ml (Rapid Polymyxin NP Test. Sensitivity 98.3%, specificity 95.4%) have been described to be used in these techniques with excellent performance. However, its main disadvantage is the requirement of additional reagents and a proper pH adjustment, making them laborious and difficult to implement in the daily routine. Even though we did not test the colistin stability in agar, other authors have reported that it can be used up to a week without affecting the results, being an advantage of our method.[17]

Despite the limitations of the study, we believe the use of one single colistin concentration (3 µg/ml) to determine colistin resistance could be a good alternative to implement in the routine workflow of microbiology laboratories with limited resources, to allow not only for a better diagnosis but also to perform epidemiological studies for the surveillance of antibiotic resistance.

AUTHOR CONTRIBUTIONS

Claudia Soria-Segarra: conception and design of the study, acquisition and analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, final approval of the version. Carmen Soria-Segarra: drafting the article or revising

it critically for important intellectual content, final approval of the version. Andrade Soriano Michelle: acquisition of data and final approval of the version. Nuñez Quezada Tamara: acquisition of data and final approval of the version. Cartelle Gestal Monica: drafting the article or revising it critically for important intellectual content, final approval of the version. Gutierrez-Fernandez José: drafting the article or revising it critically for important intellectual content, final approval of the version.

CONFLICT OF INTEREST

None to declare.

DATA AVAILABILITY STATEMENT

All the data in this study are included in the manuscript.

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