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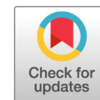

Phenotypic Detection of Plasmid-Mediated Colistin Resistance in *Enterobacteriaceae*

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ABSTRACT The aim of this work was to evaluate an easy-to-perform assay based upon inhibition of mobile colistin resistance (MCR) activity by EDTA. We included 92 nonrelated isolates of *Enterobacteriaceae* (74 *Escherichia coli*, 17 *Klebsiella pneumoniae*, and 1 *Serratia marcescens*). Our proposed method is based on a modification of the colistin agar-spot screening test (CAST), a plate containing 3 μg/ml colistin, by adding an extra plate of colistin agar-spot supplemented with EDTA (eCAST). Bacterial growth was evaluated after 24 h of incubation at 35°C. All the colistin-resistant isolates showed development on the CAST plates. Colistin-resistant *K. pneumoniae* without *mcr-1* and *S. marcescens* also grew on the eCAST plates. In contrast, colistin-resistant MCR-producing *E. coli* was not able to grow in eCAST plates. The combined CAST/eCAST test could provide a simple and easy-to-perform method to differentiate MCR-producing *Enterobacteriaceae* from those in which colistin resistance is mediated by chromosomal mechanisms.

KEYWORDS MCR, *Enterobacteriaceae*, colistin, EDTA

Worldwide dissemination of multidrug-resistant and extremely drug-resistant Gram-negative bacteria, including carbapenemase-producing *Enterobacteriaceae* led to reviving colistin (COL) as a last-resort therapy (1); this antibiotic interacts directly with the outer membrane lipopolysaccharide (2). The main resistance mechanisms involve modification of lipid A by more basic substituents; chromosome-encoded mechanisms have been known to emerge, even intratreatment, in clinically relevant microorganisms such as *Klebsiella pneumoniae* by different mutations in regulatory system genes (3–5). Since the first electronic report on the emergence of plasmid-mediated colistin resistance, including the description of the *mcr-1* (mobile colistin resistance) gene published in 2016 (6), the presence of this plasmid-dependent mechanism has been found in almost every country where it was searched for. The *mcr-1* gene encodes a phosphoethanolamine (PEtN) transferase family member, a zinc-containing metalloprotein that catalyzes the addition of PEtN to lipid A in *Escherichia coli*, conferring resistance to COL (7, 8). Even though several variants of this metalloenzyme have been described (*mcr*-2 to -*9*) (9–15), *mcr-1* is by far the most prevalent marker worldwide, where it has been disseminating unnoticed for decades.

Broth microdilution assays and the polymyxin NP test have been demonstrated to be accurate in detecting COL resistance (16, 17). However, they are not able to distinguish the COL-resistant *mcr*-producing isolates from those expressing chromosomal mechanisms (e.g., those affecting regulatory genes) (3–5). In this regard, zinc-limiting conditions have been proposed as an alternative for phenotypic identification of MCR-1-producing *E. coli* (16–19). Here, we describe an easy-to-perform phenotypic assay based upon inhibition of MCR activity by EDTA, which may enable the efficient

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TABLE 1 Results summarizing the assays of the colistin agar-spot screening test (CAST) and EDTA colistin agar-spot screening test (eCAST)

Isolate	No. of isolates	MIC_{50} and MIC range (mg/liter)	CAST[d]	eCAST[d]
COL^R *mcr* positive[a]	49	8 (4–32)	G	NG
COL^R *mcr* negative[b]	9	16 (16–64)	G	G
COL^S[c]	34	0.5 (0.25–2)	NG	NG

[a]The 49 MCR-producing isolates included 48 *E. coli* (45 *mcr-1*, 1 *mcr-2*, 1 *mcr-4*, and 1 *mcr-5*) and 1 *K. pneumoniae* (*mcr-1*); 4 out of 46 *mcr-1*-positive strains were carbapenemase producers (2 NDM-1 and 2 OXA-163).

[b]The nine colistin-resistant isolates included one *S. marcescens* and eight *K. pneumoniae*; six of them were carbapenemase producers (five KPC-2 and one NDM-1). Five out of eight *K. pneumoniae* isolates showed the Δ*mgrB* locus.

[c]The colistin-susceptible isolates included 26 *E. coli* and 8 *K. pneumoniae* (all of them *mcr* negative); 10 out of 34 were carbapenemase producers (9 NDM-1 and 1 OXA-181).

[d]G, growth; NG, no growth.

detection of MCR-producing *Enterobacteriaceae* even in resource-limited health care settings.

MATERIALS AND METHODS

A total of 92 nonrelated isolates of *Enterobacteriaceae* recovered from human ($n = 62$) and animal ($n = 30$) samples were evaluated. These included *mcr-1-like*-positive COL-resistant (COL^R) *E. coli* ($n = 45$), *mcr-2*-positive COL^R *E. coli* ($n = 1$), *mcr-4*-positive COL^R *E. coli* ($n = 1$), *mcr-5*-positive COL^R *E. coli* ($n = 1$), *mcr-1*-positive COL^R *K. pneumoniae* ($n = 1$), *mcr*-negative COL^R *K. pneumoniae* ($n = 8$), COL-susceptible (COL^S) *E. coli* ($n = 25$), COL^S *K. pneumoniae* ($n = 8$), and *Serratia marcescens* ($n = 1$), which belong to the culture collection of the Laboratorio de Resistencia Bacteriana. *E. coli* ATCC 25922 was also included. Some of the COL^R and COL^S strains are carbapenemase producers (Table 1). All isolates were previously characterized for *mcr-1* to *mcr-5* (20) and the presence of carbapenemases (21) by PCR multiplex and DNA sequencing. The *mgr*B architecture (gene encoding a negative feedback regulator of the PhoQ-PhoP signaling system) was analyzed with different PCRs using specific primers (22). Susceptibility to COL was determined by broth microdilution and interpreted following EUCAST guidelines (16).

The proposed method is based on a modification of the colistin agar-spot screening test (CAST) proposed by Servicio de Antimicrobianos, INEI ANLIS "Dr. Carlos G. Malbrán" (http://antimicrobianos.com.ar/ATB/wp-content/uploads/2017/09/Protocolo-Agar-spot-COL-2017-version2-Agosto2017.pdf), already distributed by a diagnostics company (https://www.britanialab.com/back/public/upload/productos/upl_5bd08fc36c844.pdf). In this method, a spot of approximately 10 to 15 mm is inoculated using a swab (from a 0.5 McFarland suspension) on the surface of a Mueller-Hinton agar (Britania, Argentina) plate containing 3 μg/ml COL (colistin sulfate salt; Sigma-Aldrich) (plate A). In our case, we also included an extra plate of colistin agar-spot in which EDTA (Sigma-Aldrich) was added (eCAST) (plate B: 3 μg/ml colistin Mueller-Hinton agar plus 1 mM EDTA). As a growth control, Mueller-Hinton plates with EDTA were used to show any inhibition of colony growth by EDTA itself (plate C: 1mM EDTA Mueller-Hinton agar), inoculated in the same way. The presence of colonies was evaluated after 24 h of incubation at 35°C. All assays were performed in triplicate on different dates.

In the CAST (plate A), visualization of at least 3 colonies (according to Britania's recommendations) was interpreted as COL resistance. The combination of resistance detection in plate A and lack of bacterial growth in eCAST (plate B) was interpreted as resistance to COL by MCR producers. On the other hand, bacterial growth in eCAST (≥3 colonies) was considered COL resistance without MCR production. Growth of all the tested isolates was checked in plate C for discarding inhibitory effects by EDTA alone.

The sensitivity and specificity of the combined CAST/eCAST test for detection of MCR-producing isolates was determined in comparison to the presence/absence of the *mcr* gene based on the molecular characterization of the isolates and their susceptibility profiles to COL.

Data availability. A list of the isolates tested, along with the test results, can be found at https://datadryad.org/stash/share/_g44_XaKNaudK4CMebGy1thaecK-9LRe7TNoQzST7PE.

RESULTS

We first defined the best concentration of EDTA to be incorporated into the final eCAST plates by the ability to inhibit bacterial growth only when COL resistance was due to MCR expression but not when resistance was due to chromosomal mechanisms. For these studies, seven COL^R isolates (four of them MCR producers) and three COL^S isolates were tested at 0.5 mM, 1mM, 2mM, and 5mM EDTA. As 5 mM EDTA inhibited all isolates' growth and 0.5 mM EDTA was not able to inhibit the growth of some *mcr-1*-producing isolates, a final concentration of 1 mM EDTA was chosen to prepare the B plates. These plates were used within a period of 2 months preserved at 4°C.

All COL^R isolates grew on plates of CAST (plate A); resistant *K. pneumoniae* without *mcr-1* and *S. marcescens* also displayed growth in eCAST (plate B), whereas not even a

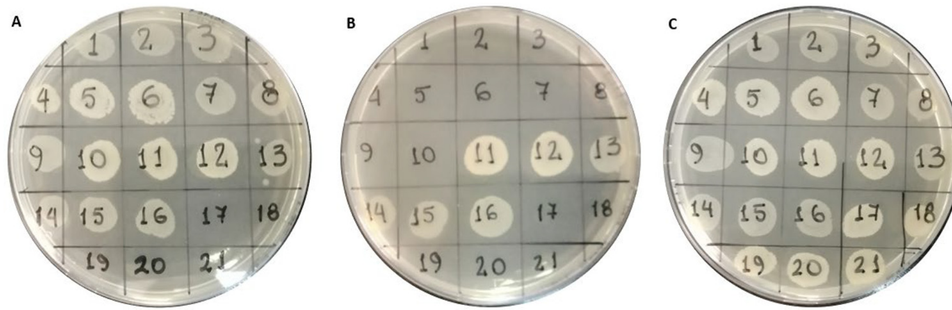

FIG 1 Differential growth in the combined CAST/eCAST test. Colistin-resistant isolates showed growth in the colistin agar-spot screening test (CAST) (plate A: Mueller-Hinton agar with 3 μg/ml COL). Of these isolates, only MCR producers did not grow in 1 mM EDTA colistin agar-spot screening test (eCAST) (plate B: Mueller-Hinton agar with 3 μg/ml COL + 1 mM EDTA). In contrast, *mcr*-negative strains harboring other resistance mechanisms also grew in these plates. A control plate (plate C: Mueller-Hinton agar with 1 mM EDTA) was used as a growth control for each isolate. Sections 1 to 10, *mcr*-positive COL-resistant isolates; 11 to 16, *mcr*-negative COL-resistant isolates; 17 to 21, *mcr*-negative COL-susceptible isolates.

single colistin-resistant MCR-producing *Enterobacteriaceae* isolate was able to grow in these plates. As expected, COL^S strains (*E. coli* and *K. pneumoniae*) exhibited no bacterial growth on both COL-containing plates. All the isolates analyzed grew in the Mueller-Hinton plates with EDTA medium (plate C). These results are exemplified in Fig. 1 and summarized in Table 1. This combined assay (plate A + plate B) showed 100% sensitivity (95% confidence interval [CI_{95}] = 92.7% to 100%) and specificity (CI_{95} = 91.8% to 100%) for the detection of MCR-producing *Enterobacteriaceae* (mostly represented by MCR-1-producing *E. coli*).

DISCUSSION

Resistance to COL, especially by plasmid-borne *mcr* genes, is being increasingly reported in bacterial isolates from humans, animals, farms, foods, and the environment. To mitigate this rapidly spreading threat, efficient and easy-to-perform diagnostic tests that allow identification of these COL^R bacteria have become indispensable and urgently necessary (23).

In this study, we evaluated a phenotypic combined CAST/eCAST test for the detection of COL-resistant MCR-positive enterobacteria recovered from human and animal samples based on the inhibition of the PEtN transferase enzyme using a chelator (EDTA). It must be noted that under the conditions described herein, standard 90-mm plates are sufficient for testing 21 isolates simultaneously, and by using the Société Française de Microbiologie 120-mm square plates, up to at least 36, which would be a clear advantage when testing large isolate collections.

The COL concentration used for the combined CAST/eCAST test was 3 μg/ml. This feature could be considered a limitation to detect the reduced number of *mcr*-harboring isolates with a COL MIC of $\leq$2 μg/ml (19), which were absent in our collection.

Previous studies for detecting MCR-harboring strains utilizing chelators such as EDTA or dipicolonic acid (DPA) have been already published. Inhibition of MCR-1 by dipicolinic acid (another metalloenzyme chelator) was reported as a useful method (called the colistin-MAC test) for the phenotypic detection of COL-resistant *E. coli*; it is a broth microdilution method displaying promising results (96.7% sensitivity and 100% specificity) for predicting *mcr-1*-positive isolates (18). Similarly, among other proposed methods that include EDTA as an inhibitor, in the colistin MIC reduction test, a COL MIC reduction in EDTA-containing wells is interpreted as MCR-1 positive, with 96.7% sensitivity and 83.3% specificity (19). In a recently modified colistin broth-disk elution test, any reduction of colistin MIC in the presence of EDTA displayed 100% and 95.8% sensitivity and specificity, respectively (24).

Finally, an EDTA-based combined disk diffusion test comparing the inhibition zones

of COL and COL plus EDTA on Mueller-Hinton agar initially proved to be useful for the detection of *mcr*-bearing *E. coli*, but further analysis showed that it produces unreliable results (25). Similarly, a DPA-based disk diffusion test was attempted with poor results. This phenomenon has been ascribed to the low diffusion of COL into the agar medium (18, 19). In this direction, we have already proposed a phenotypic assay based on COL prediffusion disks and differential inhibition with EDTA (CPD-E test) (26). In this case, however, its potential use can be foreseen as for single-isolate testing.

In conclusion, our results show that the use of the combined CAST/eCAST test could provide a simple and easy-to-perform method to differentiate colistin-resistant MCR-producing *Enterobacteriaceae* from colistin-resistant microorganisms by chromosomal mechanisms with excellent discriminatory power. It must be noted that a discrete number of different isolates can be tested in the same plates, making it more convenient for evaluating the presence of MCR in epidemiological or surveillance screenings (even in resource-limited settings) in which several strains need to be tested simultaneously without any extra (or nonconventional) equipment.

The ability to differentiate resistance mediated by *mcr* genes other than *mcr-1* opens the possibility to test natural isolates carrying these genes. This should not be taken for granted, as only one strain of each was assayed here. In any case, the tested bacteria represent the current scenario in which *mcr-1* is highly prevalent. A possibility exists that in other settings, our test may display different sensitivity and discrimination power, a general consideration that is also true for all available and newly developed methods.

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