Phenotypic detection of plasmid-mediated colistin resistance

In Enterobacteriaceae

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Running Title: Detection of \textit{mcr}-positive \textit{Enterobacteriaceae}

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ABSTRACT

The aim of this work was to evaluate an easy to perform assay based upon inhibition of MCR activity by ethylenediaminetetraacetic acid (EDTA). We included 92 non-related isolates of Enterobacteriaceae (74 E. coli, 17 K. pneumoniae and one S. 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 could also grow on the eCAST plates. In contrast, colistin-resistant MCR-producing E. coli were 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
INTRODUCTION

Worldwide dissemination of multidrug-resistant and extremely drug-resistant Gram-negative bacteria, including carbapenemase-producing Enterobacteriaceae led to reviving colistin (COL) as a last-resource 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 intra-treatment, in clinically relevant microorganisms as K. 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 was 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 addition of PEtN to lipid A in E. coli conferring resistance to COL (7,8). Even if 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 had been disseminating unnoticed for decades.

Broth microdilution assays and the polymyxin NP test have 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 ethylenediaminetetraacetic acid (EDTA), which may enable the efficient detection of MCR-producing *Enterobacteriaceae* even in resource limited health care settings.

**MATERIALS and METHODS**

A total of 92 non-related 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. pneumonia* (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 one *Serratia marcescens*, which belong to the culture collection of “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* (22) and presence of carbapenemases (23) by PCR multiplex and DNA sequencing. The *mgrB* architecture (gene encoding a negative feedback regulator of the PhoQ-PhoP signaling system) was analyzed by different PCR reactions using specific primers (24). Susceptibility to COL was determined by broth microdilution and interpreted following EUCAST guidelines (16).

In this method, a spot of approximately 10-15 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 growth control, Mueller-Hinton plates with EDTA were used to evidence any inhibition of colony growth by EDTA itself (Plate C: 1 mM EDTA Mueller-Hinton agar), inoculated in the same way. 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. Combining 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 as 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 mcr- gene based on the molecular characterization of the isolates and their susceptibility profile to COL.
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 COLR isolates (four of them MCR producers) and three COLS 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 plates B. These plates were used within a period of 2 months preserved at 4 °C.

All COLR isolates could grow 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 single colistin-resistant MCR-producing Enterobacteriaceae was able to grow in these plates. As expected, COLS strains (E. coli and K. pneumoniae) did not exhibit any bacterial growth on both COL-containing plates. All the isolates analyzed were able to grow in the Mueller-Hinton with EDTA media (Plate C). These results are exemplified in figure 1 and summarized in table 1. This combined assay (plate A + B) showed 100% sensitivity (CI95 = 92.7% – 100%) and specificity (CI95 = 91.8% – 100%) for the detection of MCR-producing Enterobacteriaceae (mostly represented by MCR-1-producing E. coli).

DISCUSSION

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.
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 identifying these COL^R^ bacteria have become indispensable and urgently necessary (25).

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 herein described conditions, 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, what 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 as a limitation to detect the reduced number of mcr-harboring isolates with COL MIC ≤ 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 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 (20).

Finally, an EDTA-based combined disk diffusion test comparing the inhibition zones of COL and COL plus EDTA on Agar Mueller-Hinton has initially proved to be useful for the detection of mcr-bearing E. coli, but further analysis showed that it produces unreliable results (21). 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 pre-diffusion 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 an 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 MCR presence in epidemiological or surveillance screenings (even in resource limited settings) in which several strains need to be tested simultaneously, without any extra (or non-conventional) equipment.

The ability to differentiate resistance mediated by other mcr genes different from 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 \textit{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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\textbf{REFERENCES}


Figure 1: Differential growth in the combined CAST/eCAST test

Colistin-resistant isolates showed growth in Colistin Agar-Spot screening test (CAST) [plate A: Agar Mueller-Hinton with COL 3μg/ml]. Of these isolates, only MCR producers did not grow in 1mM EDTA Colistin Agar-Spot screening test (eCAST) [plate B: Agar Mueller-Hinton with COL 3μg/ml + EDTA 1 Mm]. In contrasts, mcr-negative strains harboring other resistance mechanisms could also grow in these plates. Plate Control [plate C: Agar Mueller-Hinton with EDTA 1 Mm] was used as a growth control for each isolate. One to 10: mcr-positive COL resistant isolates, 11 to 16: mcr-negative COL resistant isolates and 17 to 21: mcr-negative COL susceptible isolates.
TABLE 1. Results summarizing the assays of the colistin agar-spot screening test (CAST) and EDTA colistin agar-spot screening test (eCAST)

<table>
<thead>
<tr>
<th>Isolates</th>
<th>N°</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; and MIC range (mg/L)</th>
<th>CAST</th>
<th>eCAST</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL&lt;sup&gt;a&lt;/sup&gt; mcr-positive&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49</td>
<td>8 (4-32)</td>
<td>G</td>
<td>NG</td>
</tr>
<tr>
<td>COL&lt;sup&gt;a&lt;/sup&gt; mcr-negative&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
<td>16 (16-64)</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>COL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34</td>
<td>0.5 (0.25 -2)</td>
<td>NG</td>
<td>NG</td>
</tr>
</tbody>
</table>

G: Growth; NG: No growth

<sup>a</sup>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).

<sup>b</sup>The 9 colistin-resistant isolates included 1 S. marcescens and 8 K. pneumoniae; six of them were carbapenemase-producers (5 KPC-2 and 1 NDM-1). Five out of 8 K. pneumoniae showed ΔmgrB locus.

<sup>c</sup>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).