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1 **Phenotypic detection of plasmid-mediated colistin resistance**

2 **In *Enterobacteriaceae***

3

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11

12 **Running Title:** Detection of *mcr*-positive *Enterobacteriaceae*

13

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21 **ABSTRACT**

22 The aim of this work was to evaluate an easy to perform assay based upon inhibition of
23 MCR activity by ethylenediaminetetraacetic acid (EDTA). We included 92 non-related
24 isolates of *Enterobacteriaceae* (74 *E. coli*, 17 *K. pneumoniae* and one *S. marcescens*). Our
25 proposed method is based on a modification of the Colistin Agar-Spot screening test
26 (CAST), a plate containing 3 µg/mL colistin, by adding an extra plate of Colistin Agar-Spot
27 supplemented with EDTA (eCAST). Bacterial growth was evaluated after 24 h of incubation
28 at 35°C. All the colistin-resistant isolates showed development on the CAST plates.
29 Colistin-resistant *K. pneumoniae* without *mcr-1* and *S. marcescens* could also grow on the
30 eCAST plates. In contrast, colistin-resistant MCR-producing *E. coli* were not able to grow in
31 eCAST plates. The combined CAST/eCAST test could provide a simple and easy-to-perform
32 method to differentiate MCR-producing *Enterobacteriaceae* from those in which colistin-
33 resistance is mediated by chromosomal mechanisms.

34 Keywords: MCR, *Enterobacteriaceae*, colistin, EDTA

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37 **INTRODUCTION**

38 Worldwide dissemination of multidrug-resistant and extremely drug-resistant Gram-
39 negative bacteria, including carbapenemase-producing *Enterobacteriaceae* led to reviving
40 colistin (COL) as a last-resource therapy (1); this antibiotic interacts directly with the outer
41 membrane lipopolysaccharide (2). The main resistance mechanisms involve modification
42 of lipid A by more basic substituents; chromosome-encoded mechanisms have been
43 known to emerge, even intra-treatment, in clinically relevant microorganisms as *K.*
44 *pneumoniae* by different mutations in regulatory system genes (3-5). Since the first
45 electronic report on the emergence of plasmid-mediated colistin resistance, including the
46 description of the *mcr-1* (Mobile Colistin Resistance) gene published in 2016 (6), the
47 presence of this plasmid-dependent mechanism was found in almost every country where
48 it was searched for. The *mcr-1* gene encodes a phosphoethanolamine (PEtN) transferase
49 family member, a zinc-containing metalloprotein that catalyzes addition of PEtN to lipid A
50 in *E. coli* conferring resistance to COL (7,8). Even if several variants of this metalloenzyme
51 have been described (*mcr*-2 to -9) (9-15), *mcr-1* is by far the most prevalent marker
52 worldwide, where it had been disseminating unnoticed for decades.

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

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59 MCR activity by ethylenediaminetetraacetic acid (EDTA), which may enable the efficient
60 detection of MCR-producing *Enterobacteriaceae* even in resource limited health care
61 settings.

62

63 **MATERIALS and METHODS**

64 A total of 92 non-related isolates of *Enterobacteriaceae* recovered from human (n=62) and
65 animal (n=30) samples were evaluated. These included *mcr-1-like* positive COL resistant
66 (COL^R) *E. coli* (n=45), *mcr-2* positive COL^R *E. coli* (n=1), *mcr-4* positive COL^R *E. coli* (n=1),
67 *mcr-5* positive COL^R *E. coli* (n=1), *mcr-1* positive COL^R *K. pneumoniae* (n=1), *mcr*-negative
68 COL^R *K. pneumoniae* (n=8), COL susceptible (COL^S) *E. coli* (n=25), COL^S *K. pneumoniae*
69 (n=8), and one *Serratia marcescens*, which belong to the culture collection of "Laboratorio
70 de Resistencia Bacteriana". *E. coli* ATCC 25922 was also included. Some of the COL^R and
71 COL^S strains are carbapenemase producers (Table 1). All isolates were previously
72 characterized for *mcr-1* to *mcr-5* (22) and presence of carbapenemases (23) by PCR
73 multiplex and DNA sequencing. The *mgr*B architecture (gene encoding a negative
74 feedback regulator of the PhoQ-PhoP signaling system) was analyzed by different PCR
75 reactions using specific primers (24). Susceptibility to COL was determined by broth
76 microdilution and interpreted following EUCAST guidelines (16).

77 The proposed method is based on a modification of the Colistin Agar-Spot screening test
78 (CAST) proposed by Servicio de Antimicrobianos, INEI ANLIS "Dr. Carlos G. Malbrán"
79 (http://antimicrobianos.com.ar/ATB/wp-content/uploads/2017/09/Protocolo-Agar-spot-
80 COL-2017-version2-Agosto2017.pdf), already distributed by a diagnostics company

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81 (https://www.britanialab.com/back/public/upload/productos/upl_5bd08fc36c844.pdf). In
82 this method a spot of approximately 10-15 mm is inoculated using a swab (from a 0.5
83 McFarland suspension) on the surface of a Mueller-Hinton agar (Britania, Argentina) plate
84 containing 3 µg/mL COL (Colistin sulfate salt, Sigma-Aldrich) (Plate A). In our case, we also
85 included an extra plate of Colistin Agar-Spot in which EDTA (Sigma-Aldrich) was added
86 (eCAST) (Plate B: 3µg/mL Colistin Mueller-Hinton agar plus 1 mM EDTA). As growth
87 control, Mueller-Hinton plates with EDTA were used to evidence any inhibition of colony
88 growth by EDTA itself (Plate C: 1mM EDTA Mueller-Hinton agar), inoculated in the same
89 way. Presence of colonies was evaluated after 24 h of incubation at 35 ° C. All assays were
90 performed in triplicate on different dates.

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

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

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

103 **RESULTS**

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

112 All COL^R isolates could grow on plates of CAST (Plate A); resistant *K. pneumoniae* without
113 *mcr-1* and *S. marcescens* also displayed growth in eCAST (plate B), whereas not even a
114 single colistin-resistant MCR-producing *Enterobacteriaceae* was able to grow in these
115 plates. As expected, COL^S strains (*E. coli* and *K. pneumoniae*) did not exhibit any bacterial
116 growth on both COL-containing plates. All the isolates analyzed were able to grow in the
117 Mueller-Hinton with EDTA media (Plate C). These results are exemplified in figure 1 and
118 summarized in table 1. This combined assay (plate A + B) showed 100% sensitivity (CI_{95} =
119 92.7% – 100%) and specificity (CI_{95} = 91.8% – 100%) for the detection of MCR-producing
120 *Enterobacteriaceae* (mostly represented by MCR-1-producing *E. coli*).

121 **DISCUSSION**

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122 Resistance to COL, especially by plasmid-borne *mcr* genes, is being increasingly reported in
123 bacterial isolates from humans, animals, farms, foods and the environment. To mitigate
124 this rapidly spreading threat, efficient and easy-to-perform diagnostic tests that allow
125 identifying these COL^R bacteria have become indispensable and urgently necessary (25).

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

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

136 Previous studies for detecting MCR-harboring strains utilizing chelators such as EDTA or
137 dipicolonic acid (DPA) have been already published. Inhibition of MCR-1 by dipicolinic acid
138 (another metalloenzyme chelator) was reported as a useful method (called colistin-MAC
139 test) for the phenotypic detection of COL-resistant *E. coli*; it is a broth microdilution
140 method displaying promising results (96.7% sensitivity and 100% specificity) for predicting
141 *mcr-1*-positive isolates (18). Similarly, among other proposed methods that include EDTA
142 as an inhibitor, in the Colistin MIC Reduction Test a COL MIC reduction in EDTA-containing

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143 wells is interpreted as MCR-1 positive, with 96.7% sensitivity and 83.3% specificity (19). In
144 a recently modified Colistin Broth-Disk Elution test, any reduction of colistin MIC in the
145 presence of EDTA displayed 100% and 95.8% sensitivity and specificity, respectively (20).

146 Finally, an EDTA-based combined disk diffusion test comparing the inhibition zones of COL
147 and COL plus EDTA on Agar Mueller-Hinton has initially proved to be useful for the
148 detection of *mcr*-bearing *E. coli*, but further analysis showed that it produces unreliable
149 results (21). Similarly, a DPA-based disk diffusion test was attempted with poor results.
150 This phenomenon has been ascribed to the low diffusion of COL into the agar medium
151 (18,19). In this direction, we have already proposed a phenotypic assay based on COL pre-
152 diffusion disks and differential inhibition with EDTA (CPD-E test) (26). In this case,
153 however, its potential use can be foreseen as for single isolate testing.

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

162 The ability to differentiate resistance mediated by other *mcr* genes different from *mcr-1*
163 opens the possibility to test natural isolates carrying these genes. This should not be

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164 taken for granted, as only one strain of each was assayed here. In any case, the tested
165 bacteria represent the current scenario in which *mcr-1* is highly prevalent. A possibility
166 exists that in other settings our test may display different sensitivity and discrimination
167 power, a general consideration that is also true for all available and newly developed
168 methods.

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174 harboring strains.

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180

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

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

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

282

Isolates	N°	MIC_{50} and MIC range (mg/L)	CAST	eCAST
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

283 G: Growth; NG: No growth

284 [a] The 49 MCR-producing isolates included 48 *E. coli* (45 *mcr-1*, 1 *mcr-2,* 1 *mcr-4,* and 1 *mcr-*
285 *5*), and 1 *K. pneumoniae* (*mcr-1*); 4 out of 46 *mcr-1* positive strains were carbapenemase-
286 producers (2 NDM-1 and 2 OXA-163).

287 [b] The 9 colistin-resistant isolates included 1 *S. marcescens* and 8 *K. pneumoniae*; six of
288 them were carbapenemase-producers (5 KPC-2 and 1 NDM-1). Five out of 8 *K.*
289 *pneumoniae* showed Δ*mgrB* locus.

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

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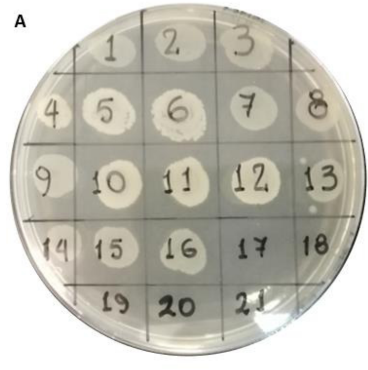
A



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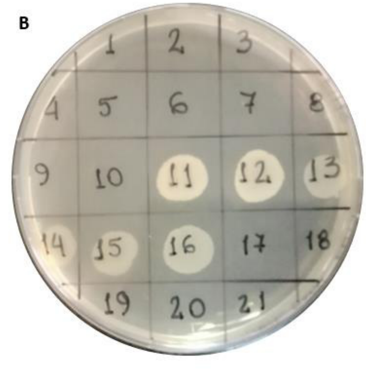

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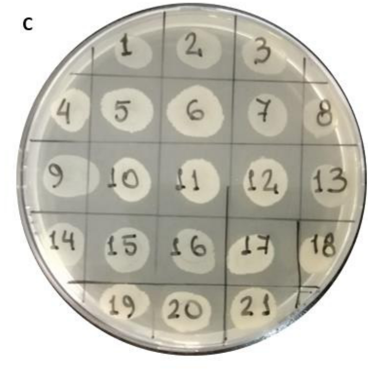

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