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# Detection of plasmid-mediated colistin resistance by colistin pre-diffusion and inhibition with EDTA test (CPD-E) in *Enterobactereaceae*

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A B S T R A C T

A phenotypic assay based on colistin pre-diffusion and differential inhibition of Mobile Colistin Resistance (MCR) protein activity by EDTA showed that, of the 92 strains tested, all MCR producers (49) exhibited an increase ≥5 mm in the inhibition zone around the area of pre-diffusion in the presence of EDTA, in comparison with colistin alone. Results suggest that CPD-E may differentiate MCR-producing microorganisms from resistant microorganisms without this marker.

## 1. Introduction

Current trends in multi-drug resistance and extreme antibiotic resistance associated with infections produced by Gram-negative bacilli led to a massive reintroduction of colistin (COL) in clinical practice (Fica et al., 2007; Yahav et al., 2012; Quiroga, Nastro and Di Conza, 2018). COL belongs to the family of polymyxins, a group of cationic polypeptides, which target is the lipopolysaccharide (LPS) rich outer membrane (Hancock and Chapple, 1999). It has been known for years that resistance could arise by target site modification, in this case lipid A, mediated by mutations in genes of the PhoPQ-PmrAB regulatory (and related) system (Blair et al., 2015; Gao et al., 2016; Giske, 2015).

It was only by the end of 2015 that the first reports on plasmid-mediated COL resistance, encoded by the *mcr-1* (Mobile Colistin Resistance) gene emerged (Liu et al., 2016). This gene encodes for a phosphoethanolamine (PEtN) transferase that catalyzes addition of PEtN to lipid A in *E. coli,* conferring resistance to COL. While other *mcr* variants have been described, *mcr-1* is still (and by far) the most widely reported plasmid resistance marker reported in all, human, animals and environmental samples (Xavier et al., 2016; Yin et al., 2017; Carattoli et al., 2017; Borowiak et al., 2017).

The ability to recognize resistance to COL due to *mcr* expression is mandatory for designing rational epidemiological surveys, and containment countermeasures (Osei, 2018). Even though classical molecular diagnostics have provided PCR assays (Lescat et al., 2018) with 100% sensitivity and specificity, there is still a need for phenotypic methods suited for clinical laboratories that are unable to perform molecular tests. Phenotypic testing to detect *mcr* producers use to be unreliable because of poor diffusion of COL in agar media, its interaction with cations and the potential adsorption of this compound to certain laboratory materials.

Crystallography studies of the catalytic domain revealed MCR is a zinc dependent metalloprotein that easily led to suggest that differential inhibition under zinc-limiting conditions should be a good alternative in the phenotypic identification of MCR-producing *E. coli* (Hinchliffe et al., 2017; Stojanoski et al., 2016).

The aim of this study was to evaluate a phenotypic assay based on pre-diffusion of colistin disk and enzymatic inhibition by ethylenediaminetetraacetic acid (EDTA) (CPD-E).

## 2. Material and methods

This work included 92 non-related isolates of *Enterobacterales*: *mcr-1* 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-1* 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* (an intrinsically resistant bacteria), already deposited at the culture collection of "Laboratorio de Resistencia Bacteriana", from different human and animal sources. *E. coli* ATCC 25922 was also included. All bacteria were recovered from their frozen stocks, isolated and fresh cultures were obtained before evaluating COL susceptibility which was determined by broth microdilution and interpreted following current EUCAST

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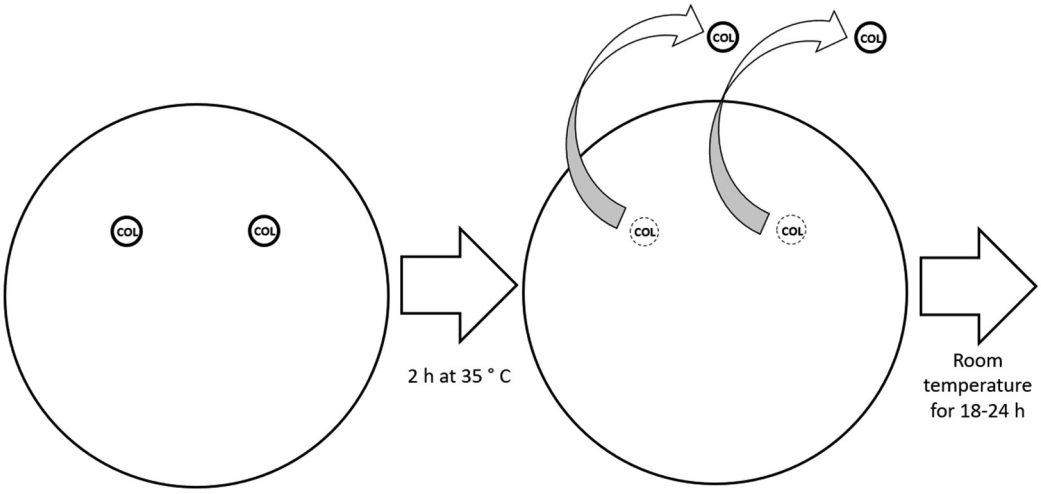
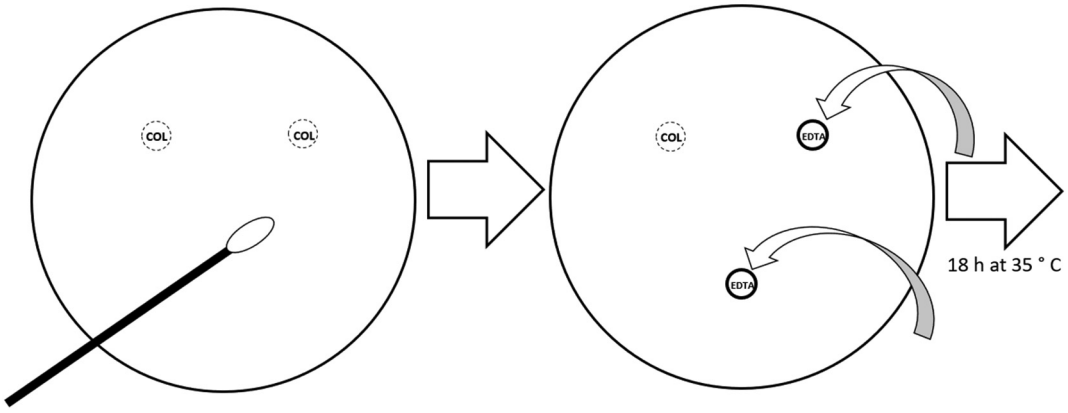



**Step 1.** On a Mueller-Hinton agar plate (without inoculation), place two colistin disks (COL), let diffuse for 2 hours at 35 ° C, remove the disks and leave the plates at room temperature for 18-24 hours.




**Step 2.** Inoculate the plate with a 0.5 Mc Farland suspension of the isolation under study and place two disks of the EDTA (1µmol), one on the diffusion site of one of the colistin disks and the other to confirm that EDTA does not have antibacterial effect.

**Fig. 1.** Colistin pre-diffusion and inhibition with EDTA (CPD-E) test.

guidelines (EUCAST, 2019). All isolates were characterized for *mcr-1* to *mcr-5* presence by PCR-multiplex using specific primers (Lescat et al., 2018).

The test was carried out as follows: two COL disks (10 µg, Britania, Argentina) were placed and allowed to diffuse for 2 h at 35 °C on Mueller-Hinton (MH) agar (Britania, Argentina) plate (25 mL on 9 cm diameter plates). Then, disks were removed and the plates left at room temperature for 18–24 h. At that time, plates were inoculated using the standard conditions with a 0.5 of McFarland bacterial suspension, and two disks containing 1 µmol of EDTA (Sigma-Aldrich) (pH 8.0) were placed, one on the diffusion site, exactly where the COL disk had been originally located, and the other anywhere else, at least 30 mm away from each other, in order to confirm that EDTA has no inhibitory effect on its own, as it could happen at higher EDTA concentrations (Fig. 1). Diameters of inhibition zones around COL and these EDTA disks were measured after 18 h of incubation at 35 °C. The assay was performed in triplicate on different dates, using independently obtained inoculum in each case.

## 3. Results and discussion

All $COL^R$ *E. coli* and *K. pneumoniae* isolates that were MCR producers ($n$ = 49, MIC > 2 µg/mL – EUCAST), showed an increase ≥5 mm (Δ between 5 and 16 mm) in the inhibition zone around the area of pre-diffusion in the presence of EDTA, in comparison with inhibition areas in the absence of EDTA. No inhibition could be detected around the EDTA control disks in any case. $COL^S$ *E. coli*, $COL^S$ *K. pneumoniae*, $COL^R$ *K. pneumoniae* lacking *mcr* and *S. marcescens* ($n$ = 43), displayed a negligible or nil difference in the diameters (Δ = 0–3 mm). Results are shown in Supplementary Table 1 and summarized in Fig. 2. Analysis was performed by plotting the inhibition zone increase between MCR-1-producing isolates and non MCR-producing *Enterobacterales* (Fig. 3). Statistical analysis showed a significant difference using a cutoff value of at least 5 mm [$p$-value < 0.05 (test Mann-Whitney-Wilcoxon)]. Under these conditions, the sensitivity and specificity of CPD-E were both 100% so far (CI95 = 92.1%–100% and CI95 = 91.8% –100%, respectively), indicating a very good test with a high discriminative power.

Results from the isolates containing *mcr-2*, *mcr-4* and *mcr-5* were similar, but they were not included in the scatterplot, as they are not clinical isolates from our geographic area and may introduce some bias in our analysis. The MCR-1 producing *K. pneumoniae* was collected in our area, but as it is a single isolate (as already known, this is a rare event in acquired resistance in this species) we also want to be cautious

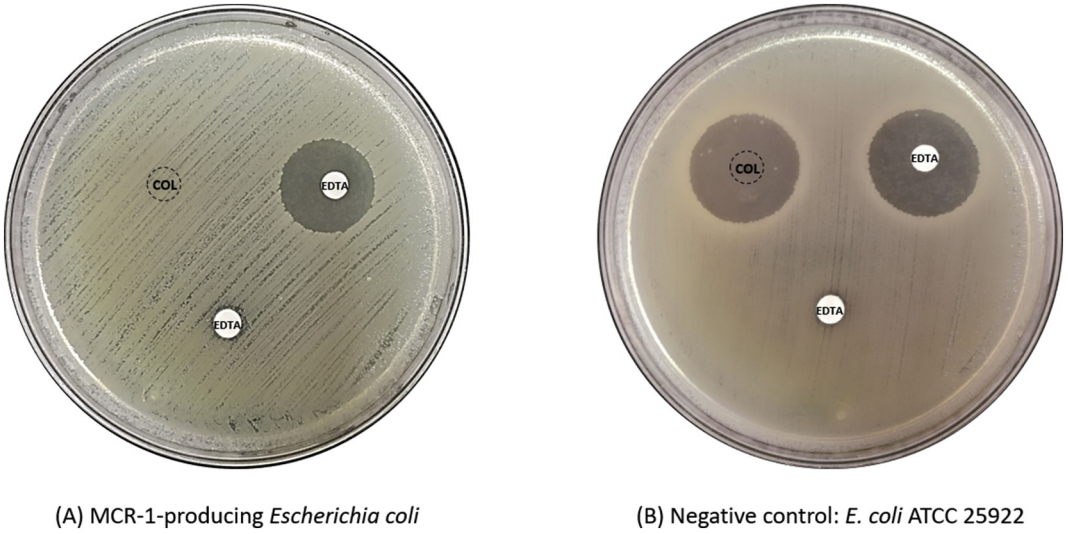



**Fig. 2.** (A) Colistin-resistant isolates MCR-1-producing showed an increase ≥5 mm in the inhibition zone around the area of pre-diffusion of colistin in the presence of EDTA. (B) Colistin- susceptible isolates (*E. coli* ATCC 25922), displayed a negligible or nil difference in the inhibition zone around the area of pre-diffusion of colistin with and without of EDTA.

before making a generalization. In any case, their inclusion shows that the same method may also detect their presence.

Recently, the inhibition of MCR-1 by dipicolinic acid (another chelator) was published as a useful method (called colistin-MAC test) for the phenotypic detection of colistin-resistant *mcr-1*-harboring *E. coli*, through broth microdilution (BMD) method displaying promising results (96.7% sensitivity and 100% specificity) for predicting *mcr-1*-positive isolates (Coppi et al., 2018).

Other methods propose EDTA as an inhibitor. Among them, in the Colistin MIC Reduction Test (CMR), a COL MIC reduction in EDTA-containing wells is interpreted as MCR-1 positive, with 96.7% sensitivity and 83.3% specificity. The Zeta Potential Alteration, based in Zeta potential ratio (Rzp) calculated in the presence and absence of EDTA, has 95.1% sensitivity and 100% specificity (both BMD tests) (Esposito et al., 2017).

Finally, in the Combined Disk Test ± EDTA (CDT), an incremental difference of ≥ 3 mm between a non-pre-diffused colistin disk and a colistin + EDTA disk is interpreted as MCR-1-positive, with 96.7% and 89.6% of sensitivity and specificity, respectively (Esposito et al., 2017). In this case, however, Clement et al. obtained a sensitivity of < 68%, concluding that CDT is unreliable for *mcr-1*-producing *Enterobacteriaceae* detection and that this phenomenon can be ascribed to the low diffusion of COL into the agar medium (Clément et al., 2018).

The pre-diffusion technique described in this work yielded in a much larger difference between inhibition zones of COL with EDTA and COL alone defining a cut-off value of at least 5 mm which provided a

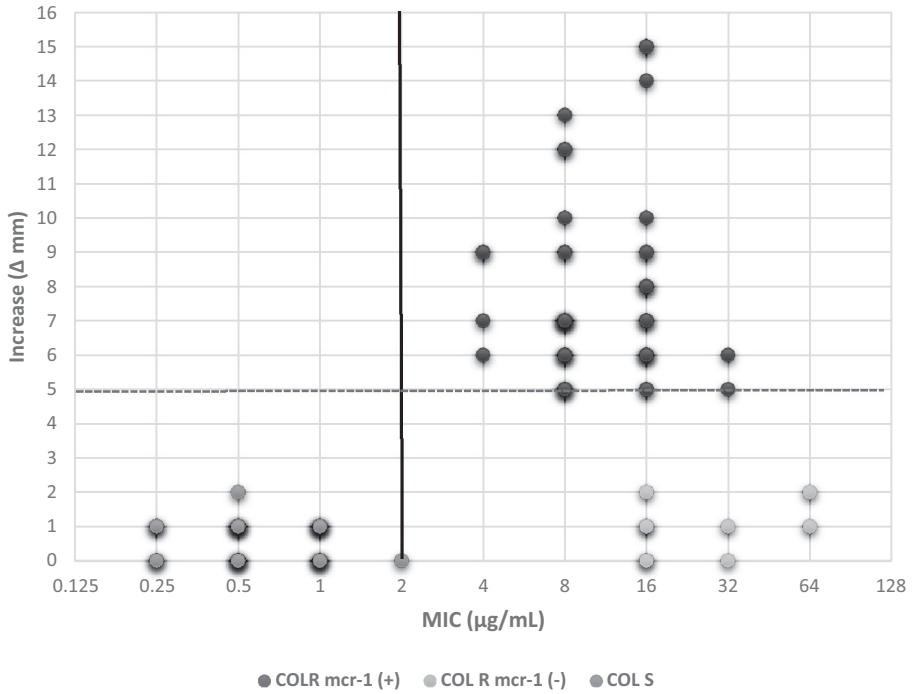



**Fig. 3.** Scatter-Plot between the increase of inhibition zone in the area of pre-diffusion of COL with and without EDTA versus COL MICs. The size of each dot is proportional to the number of isolates.

100% sensitivity and specificity for *mcr* detection.

In conclusion, results of this work suggest that CDP-E could provide a very simple to perform method in order to detect MCR-producing *Enterobacteriaceae* in low complexity diagnostic laboratories. It can be useful especially on conventional epidemiological surveys, providing a friendly and non-expensive test. Additional studies with greater isolate numbers, under varied epidemiological situations are, obviously, still necessary to validate on a large scale this methodology accuracy.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105759.

## Declaration of Competing Interest

No conflict of interest declared.

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