UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
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# Avaliação do EDTA na microdiluição em caldo com polimixina B como teste fenotípico para detecção do gene *mcr*-1

Aline Gabrielle Alves Nunes

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Área de habilitação: Microbiologia Clínica

Orientadora: Dra. Priscila Lamb Wink

Coorientador: Prof. Dr. Afonso Luís Barth

Porto Alegre, dezembro de 2018.

**Dedicatória**

Ao meu marido e à minha filhinha de quatro patas, que compartilharam essa caminhada comigo do início ao fim.

“A maravilhosa disposição e harmonia do universo só pode ter tido origem segundo o plano de um Ser que tudo sabe e tudo pode. Isso fica sendo a minha última e mais elevada descoberta.”
-Isaac Newton

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Este trabalho foi escrito segundo as normas do “Brazilian Journal of Microbiology”, apresentadas em anexo (Anexo 1).

**Title:** Evaluation of EDTA in the broth microdilution using polymyxin B as a phenotypic test to detect the *mcr*-1 gene

**Running title:** EDTA as a phenotypic test for *mcr*-1

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**Abstract**

Polymyxins are antibiotics that have recently regained significant interest as a consequence of the increasing infections due to multidrug-resistant Gram-negative bacteria. Currently, colistin and polymyxin B are used as last-line drugs for treating bacterial infections. However, polymyxin resistance has increased, and knowledge of its mechanisms is expanding. We aimed to evaluate the reduction of polymyxin MIC value based on the inhibition of the MCR-1 activity by the chelator ethylenediaminetetraacetic acid (EDTA) in the broth microdilution (BMD) method in presence of 2 mM. All *mcr*-1 positive isolates (n = 47) displayed a decrease in the MIC value for polymyxin B with the addition of EDTA, where 96% (n=45) displayed a decrease of at least 2-fold dilutions. Two *mcr*-1 positive isolates presented a decrease of only 1-fold dilution with EDTA. None of the *mcr*-1 negative (n = 28) isolates presented significant decrease in MIC values when the chelator was added to polymyxin B. Our results indicate that inhibition of MCR-1 by EDTA can be used for the presumptive detection of MCR-1-producing bacteria isolates when the BMD assay is applied.

Keywords: polymyxin resistance; *mcr*-1; susceptibility test; EDTA.

## 1. Introduction

Colistin (polymyxin E) and polymyxin B belong to a group of polypeptide antibiotics classified as polymyxins, which have recently regained significant interest as a consequence of the increasing incidence of infections due to multidrug-resistant Gram-negative bacteria. Currently, colistin and polymyxin B are used as last-line drugs for treating severe infections caused by multidrug-resistant Gram-negative pathogens, in particular the carbapenem-resistant *Enterobacterales*. However, polymyxin resistance has increased gradually for the last few years, and knowledge of its multifaceted mechanisms is expanding (Falagas et al., 2005; Kaye et al.,2016; Poirel et al., 2017).

The intrinsic resistance in some Gram-negative species such as *Proteus* spp., *Providencia* spp., *Morganella* spp., *Serratia* spp., and *Burkholderia* spp. is associated to the expression of genes that increase the positive charge of the lipopolysaccharides, which reduces the action of polymyxins. Nevertheless, recently the acquisition of polymyxin resistance has been rising (Olaitan et al., 2014). In November 2015, Liu first described a gene responsible for the horizontal transfer of resistance to polymyxins, mediated by plasmid, known as *mcr*-1 (Liu et al., 2016). The production of MCR-1, the protein encoded by the gene *mcr*-1 gene, leads to an increase in the minimum inhibitory concentration (MIC) for polymyxins (Poirel et al., 2017), and is a major worldwide health problem as it may disseminate rapidly.

Recent studies have demonstrated that the structure of the catalytic site of the phosphoethanolamine transferase MCR-1, is a zinc metalloprotein and the addition of a chelator such as ethylenediaminetetraacetic acid (EDTA) in the broth microdilution method (BMD) for polymyxin susceptibility testing may reduce colistin MICs in *E. coli* strains expressing the *mcr*-1 gene (Hinchliffe et al., 2017, Esposito et al., 2017).

Due to the increasing emergence of polymyxin resistance, the development of specific screening methods to determine the susceptibility and resistance to polymyxins is an urgent need for clinical laboratories in order to improve patient therapeutics and prevent dissemination of the *mcr*-1 gene. In this study, we evaluated the effect of polymyxin B MIC based on the inhibition of the MCR-1 activity by EDTA in the BMD with the presence of EDTA (2 mM).

## 2. Materials and Methods

### 2.1 Isolates

A total of 75 isolates were tested: 47 *mcr*-1 positive (36 *Escherichia coli*, 2 *Klebsiella pneumoniae*, 9 *Salmonella enterica*) and 28 *mcr*-1 negative (*K. pneumoniae*). All isolates included in this study were obtained from previous surveillance studies of clinical and food-producing animals in southern Brazil from 2013 to 2018.

### 2.2 Identification of the *mcr*-1 gene

The presence of the *mcr*-1 gene was evaluated by submitting isolated colonies to DNA extraction followed by conventional PCR. Briefly, thermal lysis was carried out for DNA extraction where pure isolated colonies were transferred to an extraction buffer (TE) and subjected to heating at 80 °C for 20 minutes, followed by freezing at -20 °C for 20 min. The extracted DNA was submitted to conventional PCR with specific primers for the *mcr*-1 gene (forward, 5′-CGGTCAGTCCGTTTGTTC-3′; and reverse, 5′-CTTGGTCGGTCTGTAGGG-3′) (Liu et al., 2016).

### 2.3 EDTA sub inhibitory concentration

Prior to perform the BMD with polymyxin B and EDTA, a BMD assay without antibiotic was carried out varying the EDTA concentration from 64 mM to 0.125 mM in order to establish the lowest sub inhibitory concentration of EDTA for the isolates included in the study.

### 2.3 Broth microdilution method (BMD)

After we established the EDTA sub inhibitory concentration for the *mcr-1* positive and *mcr-1* negative isolates, polymyxin MICs were determined by BMD according to ISO 20776-1:2006 procedures. Results were interpreted according to the *European Committee on Antimicrobial Susceptibility Testing* guidelines (EUCAST, 2018), i.e., values of MIC > 2 µg/mL were considered resistant. Polymyxin MIC reduction was evaluated in the presence of EDTA at a final concentration of 2 mM. All microdilution tests were performed in duplicate. The *E. coli* ATCC 25922 was used as quality control. A significant MIC decrease was considered when at least 2-fold dilutions of polymyxin MIC with EDTA were observed.

## 3. Results

The lowest sub inhibitory concentration of EDTA was 2 mM for all isolates. A total of 45 *mcr*-1 positive isolates (96%) displayed a significant decrease in the MIC value for polymyxin B with the addition of EDTA. Two *mcr*-1 positive isolates presented a decrease of MIC of only 1-fold dilution with EDTA. None of the *mcr*-1 negative isolates (n = 28) presented significant difference in MIC values when the chelator was added to polymyxin B (Table 1).

## 4. Discussion

The reference method for the susceptibility analysis of a bacterial against polymyxins is the BMD. Despite being a laborious procedure, which requires reagents which may be expensive (antibiotic salt), it is a reliable and accurate assay (Humphries, 2015).

The lowest sub inhibitory concentration of EDTA in the BMD was fixed at 2 mM, since this concentration showed no antibacterial activity against all screened isolates. Moreover, our results in the BMD with 2 mM EDTA demonstrate that inhibition of the *mcr*-1 gene by EDTA occurs and this approach can be used for the presumptive detection of MCR-1-producing bacteria isolates in diagnostic laboratories. We observed a decrease of at least 2-fold in the MIC value for polymyxin B in the presence of 2 mM EDTA for the vast majority (96%) of *mcr*-1 positive isolates. On the other hand, the MIC values for the *mcr*-1 negative isolates did not change significantly, which confirms the specificity of EDTA as an inhibitor of the MCR-1 protein.

Other approaches have been tested to evaluate the effect of EDTA in the inhibition of the MCR-1. In a recent study by Esposito and colleagues (Esposito et al., 2017), distinct phenotypic tests were evaluated for the detection of colistin-resistant MCR-1-positive *E. coli* based on the inhibition of the MCR-1 phosphoethanolamine transferase by EDTA, including a combined-disk test (CDT) comparing the inhibition zones of colistin and colistin (10 µg) plus EDTA (100 mM); reduction of colistin MIC (CMR) in the presence of EDTA (80 µg g/mL); and a modified rapid polymyxin Nordmann/Poirel test (MPNP). They obtained encouraging results for the detection of MCR-1 in *E. coli* isolates using the following assay parameters: ≥3 mm difference in the inhibition zones between colistin disks without and with EDTA; ≥4-fold colistin MIC decrease in the presence of EDTA; and the absence of metabolic activity and

growth, indicated by unchanged color of phenol red in the presence of colistin-EDTA, in the MPNP test.

Similarly, molecular and structure studies of the catalytic domain of MCR-1 protein have supported that phosphoethanolamine transferase can be assigned as a member of the alkaline phosphatase metalloenzyme superfamily, with zinc being required for MCR activity (Sun et al., 2017; Hinchliffe et al., 2017; Stojanoski et al., 2016). Indeed, for MCR-1-positive strains, a significant decrease in colistin MIC was observed in the presence of EDTA, supporting the idea that zinc-limiting conditions induced by EDTA represent a good alternative for phenotypic identification of MCR-1-producing bacteria (Hinchliffe et al., 2017). Recently, the inhibition of MCR-1 by an another metalloenzyme chelator, dipicolinic acid, was reported as a useful method for phenotypic screening of *mcr*-1-positive colistin resistant *E. coli* strains (Coppi et al., 2018).

It has to be considered that EDTA has been shown to be effective against *mcr*-1 positive isolates with low concentration of MIC. It would be important to test *mcr*-1 positive isolates with high concentration MICs to confirm the inhibition of MCR-1 by a metalloenzyme chelator; however *mcr*-1 isolates with high MICs are very rare. On the other hand, we tested *mcr*-1 negative isolates with low and high concentration of MIC and we did not observe any significantly differences, confirming the specificity of EDTA as an inhibitor of the MCR-1 protein.

In conclusion, our results indicate that inhibition of MCR-1 by EDTA is a promising approach, which can be used for the presumptive detection of MCR-1-producing bacteria using the BMD.

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**Conflict of interest:**

All authors report no conflicts of interest relevant to this article.

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**Table 1.** List of isolates used in the BMD and alteration of the MIC values for the *mcr-1* positive isolates with addition of 2 mM EDTA.

| Isolates | Species | MIC | | MIC fold change[c] |
|---|---|---|---|---|
| | | Without EDTA | With EDTA 2mM | |
| ***mcr-1* positive:** | | | | |
| 3431F | *E. coli* | 4 | 0.25 | 4 |
| 234 | *E. coli* | 2 | 0.25 | 3 |
| 5798F | *E. coli* | 4 | 0.25 | 4 |
| 6699F | *E. coli* | 4 | 0.25 | 4 |
| 16879 | *E. coli* | 4 | 0.25 | 4 |
| 16938 | *E. coli* | 2 | ≤0.125 | 4 |
| 16944 | *E. coli* | 2 | 0.25 | 3 |
| 15088 | *E. coli* | 4 | 0.25 | 4 |
| 13300 | *E. coli* | 2 | 0.5 | 2 |
| 13478 | *E. coli* | 2 | ≤0.125 | 4 |
| 13891 | *E. coli* | 2 | 0.25 | 3 |
| 17428 | *E. coli* | 4 | 0.5 | 3 |
| 17252 | *E. coli* | 2 | 0.25 | 3 |
| 17425 | *E. coli* | 2 | 0.5 | 2 |
| 17426 | *E. coli* | 4 | 0.5 | 3 |
| 17217 | *E. coli* | 2 | 0.25 | 3 |
| 17185 | *E. coli* | 2 | 0.5 | 2 |
| 18814 | *E. coli* | 2 | 0.25 | 3 |
| 17084 | *E. coli* | 2 | 0.5 | 2 |
| 18636 | *E. coli* | 4 | 0.5 | 3 |
| 14460 | *E. coli* | 4 | 0.5 | 3 |
| 14462 | *E. coli* | 2 | 0.5 | 2 |
| 17877 | *E. coli* | 2 | 0.5 | 2 |
| 16452 | *E. coli* | 2 | 0.25 | 3 |
| 16720 | *E. coli* | 2 | 0.5 | 3 |
| 14571 | *E. coli* | 2 | 0.25 | 3 |
| 14937 | *E. coli* | 2 | 0.5 | 2 |
| 15486 | *E. coli* | 2 | 0.5 | 2 |
| 15487 | *E. coli* | 2 | 0.25 | 3 |
| 15784 | *E. coli* | 1 | 0.25 | 2 |
| 15796 | *E. coli* | 2 | 0.25 | 3 |
| 14065 | *E. coli* | 2 | 1 | 1 |
| 14071 | *E. coli* | 2 | 0.5 | 2 |
| 14459 | *E. coli* | 4 | 0.5 | 3 |
| 16408 | *E. coli* | 2 | 0.25 | 3 |
| 17256 | *E. coli* | 2 | 0.5 | 3 |
| 3111F | *K. pneumoniae* | 4 | 0.25 | 4 |

| Isolates | Species | MIC | | MIC fold change[c] |
|---|---|---|---|---|
| | | Without EDTA | With EDTA 2mM | |
| 18425 | *K. pneumoniae* | 8 | 4 | 1 |
| 6480/16 | *S. enterica* | 8 | 0.5 | 4 |
| 940/15 | *S. enterica* | 4 | 0.5 | 3 |
| 7313/15 | *S. enterica* | 4 | 0.5 | 3 |
| 7572/15 | *S. enterica* | 4 | 0.5 | 3 |
| 6310-15 | *S. enterica* | 2 | 0.5 | 2 |
| 5538-17 | *S. enterica* | 4 | 0.25 | 4 |
| 3383/15 | *S. enterica* | 4 | 0.25 | 4 |
| 0938/15 | *S. enterica* | 2 | 0.25 | 3 |
| 6297/15 | *S. enterica* | 2 | 0.5 | 2 |
| ***mcr-1* negative:** | | | | |
| 4453 | *K.pneumoniae* | 16 | 8 | 1 |
| 4176 | *K.pneumoniae* | 2 | 4 | 1 |
| 2089 | *K.pneumoniae* | 16 | 8 | 1 |
| 3513 | *K.pneumoniae* | 4 | 8 | 1 |
| 3514 | *K.pneumoniae* | 16 | 8 | 1 |
| 1762 | *K.pneumoniae* | 2 | 4 | 1 |
| 1957 | *K.pneumoniae* | 4 | 4 | - |
| 78 | *K.pneumoniae* | 8 | 4 | 1 |
| 908 | *K.pneumoniae* | 16 | 16 | - |
| 966 | *K.pneumoniae* | 64 | 32 | 1 |
| 1188 | *K.pneumoniae* | 32 | 16 | 1 |
| 889 | *K.pneumoniae* | 64 | 64 | - |
| 4178 | *K.pneumoniae* | 16 | 16 | - |
| 4447 | *K.pneumoniae* | 16 | 16 | - |
| 4090 | *K.pneumoniae* | 4 | 4 | - |
| 3527 | *K.pneumoniae* | 8 | 8 | - |
| 3742 | *K.pneumoniae* | 16 | 8 | 1 |
| 3854 | *K.pneumoniae* | 32 | 16 | 1 |
| 2242 | *K. pneumoniae* | 32 | 32 | - |
| 2076 | *K. pneumoniae* | 32 | 32 | - |
| 366 | *K. pneumoniae* | 2 | 2 | - |
| 1519 | *K. pneumoniae* | 8 | 8 | - |
| 1520 | *K. pneumoniae* | 0.5 | 0.5 | - |
| 2889F | *K. pneumoniae* | 8 | 4 | 1 |
| 2695F | *K. pneumoniae* | 16 | 16 | - |
| 2702F | *K. pneumoniae* | 0.25 | 0.25 | - |
| 2924F | *K. pneumoniae* | 0.5 | 1 | 1 |
| 3268F | *K. pneumoniae* | 4 | 2 | 1 |

[c]Dashes in empty cells indicate no MIC reduction/increase (fold change) in the presence of 2 mM EDTA.

**ANEXO I – Normas de publicação da revista "BRAZILIAN JOURNAL OF MICROBIOLOGY"**

## GUIDE FOR AUTHORS

### INTRODUCTION

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### SECTIONS

Biotechnology and Industrial Microbiology: Biosynthesis and bioconversion of natural products, including antibiotics, xenobiotics, and macromolecules produced by bacteria. Biosynthesis and bioconversion of natural products, including antibiotics, xenobiotics, and macromolecules produced by fungi. Molecular aspects of fungal biotechnology. Molecular aspects of bacterial biotechnology. Food Microbiology: Applications of microorganisms (bacteria and fungi) for food production. Food borne diseases, food spoilage, and microbial ecology in foods. Bacterial and Fungal Pathogenesis: The genetic, biochemical, and structural basis of bacterial pathogenesis. Clinical Microbiology: Studies of medically-important bacteria, fungi and virus. Environmental Microbiology: Ecology of natural microbial assemblages, microbial diversity of natural environments such as water, soil, sediments and higher organisms. Microbial interactions. Biodegradation, Bioremediation, and Environmental considerations for genetically engineered microorganisms. Veterinary Microbiology: Diseases of animals, Control and/or treatment of animals, Animal pathogen diagnostics, and Veterinary or zoonotic pathogens Fungal and Bacterial Physiology: Biochemistry, biophysics, metabolism, cell structure, stress response, growth, differentiation and other related process. Bacterial, Fungal and Virus Molecular Biology: Fungal and bacterial genetics, molecular biology, gene regulation, DNA replication and repair, genomics, proteomics, transcriptomics. Education in Microbiology: Original research papers and

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Either British or American spelling is accepted if used consistently throughout the manuscript. Authors who are not native English speakers are required to send a certificate that the article had its language reviewed by a professional English editing service. This certificate must be attached to the submission as a supplementary file. The quality of the language is the author's responsibility. All manuscripts will undergo copyediting before printing, but significant language editing will not be undertaken. Poor quality of English masks the possible scientific merit of some papers, and can delay publication or lead to outright rejection of the paper.

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