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ORIGINAL ARTICLE

Identification of Colistin-resistant *Enterobacteriacae* and Assessment of DPA microdilution test for Phenotypic Detection of *mcr* gene

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ABSTRACT

Background: *Colistin resistance presents a challenge for microbiology laboratories, where broth microdilution (BMD) was recommended to be the standard method for testing the susceptibility of gram-negative bacteria. However, that phenotypic test is incapable to discriminate colistin resistance attributed to the gene of mcr-1 as opposed to chromosomal mechanisms of resistance. Adding a metallo-chelator agent like dipicolinic acid (DPA) to the BMD of colistin could potentially decrease the minimum inhibitory concentrations (MICs) of colistin for isolates that express the gene mcr-1.* ***Objective:*** *The study aims to estimate the frequency of colistin resistant Enterobacteriaceae and to evaluate the impact of DPA on MIC of colistin as determined by BMD.* ***Methodology:*** *This study was done on a total of 71 Enterobacteriaceae isolates. Confirmation of isolates and detection of colistin resistance was done then phenotypic detection of plasmid-mediated mcr-1 gene was done by measuring colistin MIC using DPA in BMD.* ***Results:*** *Our study revealed that 74.6% of the isolates were colistin resistant, 7.4% of the isolates possessed mcr-1 gene. When DPA was added, all strains carrying the mcr-1 exhibited a decrease in the MIC of colistin by a minimum eightfold dilution (sensitivity 100%). However, 51% of the mcr-1 negative strains showed a similar reduction of MIC (specificity 48.98%). This test presented accuracy of 52.83%.* ***Conclusion:*** *DPA-based microdilution test is not a sufficiently accurate test to rely on in the identification of the mcr-1 producers. However, it is a highly sensitive test, it could be used as an effective method to screen for mcr-1 presence.*

INTRODUCTION

Antibiotic resistance in Gram-negative bacteria is a significant global issue. Polymyxins serve as the ultimate choice in combating highly resistant Gram-negative bacteria [1]. Colistin, a polycationic antibiotic, is highly efficient against Gram-negative bacteria, including *Enterobacteriaceae*. Its primary target is the outer cell membrane of bacteria, where it interacts with lipopolysaccharides. Due to this interaction, lipopolysaccharides are disrupted increasing membrane's and ultimately resulting in cell death [2,3].

Initially, polymyxin resistance was thought to be associated only with chromosomal mechanisms. Nevertheless, in 2016, bacterial isolates were found to have a gene related to plasmid-mediated resistance, that was named as mobile colistin resistance -1 (*mcr-1*). This gene encodes an enzyme in charge of phosphoethanolamine synthesis and conjugation to lipid A, which results in resistance to colistin [4]. The presence of this resistance gene implies the existence of various pathways for the horizontal transmission of colistin resistance, which can cause a wide range of multidrug-resistant phenotypes in bacteria that affect humans and animals [5].

Microbiology laboratories continue to have difficulties when testing for colistin resistance. The broth microdilution (BMD) test is the reference method recommended by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility testing (EUCAST) for assessing the susceptibility[6]. This phenotypic test, however, is unable to differentiate between resistance to polymyxin caused by chromosomal mechanisms and plasmid mediated mechanisms [1].

Studies have shown that *mcr-1* encodes phosphoethanolamine transferase consisting of a zinc metalloprotein. The introduction of a metallo-chelator, such as ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid (DPA), into the BMD of polymyxins might lead to a decrease in the minimum inhibitory concentration (MIC) of colistin for isolates expressing that gene [7].

The study aims to estimate the frequency of colistin resistant *Enterobacteriaceae* and to evaluate the impact of DPA on MIC of colistin as determined by BMD.

METHODOLOGY

Our cross-sectional study involved 71 *Enterobacteriaceae* isolates (48 *E. coli* and 23 *Klebsiella* spp.) acquired from the strain bank of Cairo University, Faculty of Medicine, Medical Microbiology and Immunology Department. The original source of these isolates was urine samples collected from inpatients at Kasr Al-Ainy Hospitals. The research protocol granted an approval from the Ethical Committee of the Faculty of Medicine at Cairo University (N-452-2023).

Identification of isolates

Conventional microbiological techniques such as colony morphology, Gram stained smears, glucose fermenting, and oxidase testing were used to validate the isolates. Those that were identified as rose-pink colonies on MacConkey's agar indicating lactose fermentation, glucose fermenters and oxidase-negative as well as being Gram negative bacilli were considered as *Enterobacteriaceae*. Conventional biochemical assay, including triple sugar iron agar (TSI), citrate test, motility indole ornithine (MIO) and urease test, were employed to identify the species of the isolates[8].

Phenotypic detection of colistin resistance by BMD and effect of addition of DPA on colistin MIC:

The BMD technique was used to measure colistin MIC. The concentration ranges of colistin-sulphate (Sigma-Aldrich, USA) were 128 µg/ml to 0.25 µg/ml both independently and in conjunction with DPA at 900 µg/ml constant concentration. Dimethyl sulphoxide (DMSO) (SERVA, Heidelberg, Germany) was used to formulate the stock solution of DPA (Sigma-Aldrich) at a concentration of 100 mg/ml. For growth control, Mueller-Hinton broth was utilized, with 900 mg/ml final concentrations of DMSO and DPA. The results were documented after an incubation period of 24 hours at 35°C. In the outline of CLSI recommendations, colistin MICs of less than or equivalent 2 µg ⁄ml were considered sensitive, whereas MICs more than or equal 4 µg ⁄ml were considered resistant [9]. All BMD tests were conducted in duplicate. The strain of *E. Coli* that is susceptible to colistin (ATCC 25922), served as a quality control in this study. A reduction of MIC of at least 2 folds when of DPA was added was considered a significant result.

Detection of plasmid-mediated *mcr-1* gene:

The *mcr-1* gene identification was carried out in the Molecular Biology Unit of Cairo University's Faculty of Medicine's Medical Biochemistry Department. The extraction of DNA template was carried out using DNA Mini Kit QIAamp (Qiagen, Germany, cat. no. 51306) in compliance with the guidelines provided by the instructor. This was followed by amplification by conventional polymerase chain reaction (PCR) utilizing primers that were selected from previously published sequences as displayed in Table 1 and detection by gel electrophoresis (Biometra, Germany). The visual representation of the anticipated bands of DNA at 320 kb indicated a positive isolate carrying the *mcr-1* gene.[10].

Table 1: Primers employed for amplification of *mcr-1* gene

Primer name	Sequence (5'-3')	Target gene	Amplicon Size (bp)	Working conc. Per reaction tube of each primer
*mcr1*_fw	AGTCCGTTTGTTCTTGTGGC	*mcr-1*	320	10 pmol
*mcr*1_rev	AGATCCTTGGTCTCGGCTTG			

mcr: mobile colistin resistance; fw: forward; rev: reverse [10]

Statistical Analysis

The data was entered and coded utilizing SPSS (Statistical Package for the Social Sciences) version 28 (IBM Corp, Armonk, NY, USA). Quantitative data was done by computing the measures of central tendency. A Chi-square ($\chi 2$) test was employed to compare categorical data with an exact test used when the expected frequency was below 5. The standard diagnostic indices were calculated following the method described by ***Galen*** [12]. Statistical significance was set at *p-value* less than 0.05.

RESULTS

In this study, a total of 71 isolates of *Enterobacteriaceae* were included, retrieved from 28 (39.4%) males and 43 (60.6%) females. They were between the age of 2 and 91 years with a mean of 46.86 ± 24.17 years. All isolates were obtained from urine samples. The isolates were determined as 48 (67.6%) *E. coli* and 23 (32.4%) *Klebsiella pneumonia*.

BMD method for colistin resistance determination:

Using the BMD technique, it was found that of the 71 isolates, 53 (74.6%) were resistant to colistin and 18 (25.4%) were susceptible to it as illustrated in figure 1.

The resistance rate was 66.7% (32/48) among *E. coli* and 91.3% (21/23) among *Klebsiella pneumoniae*.

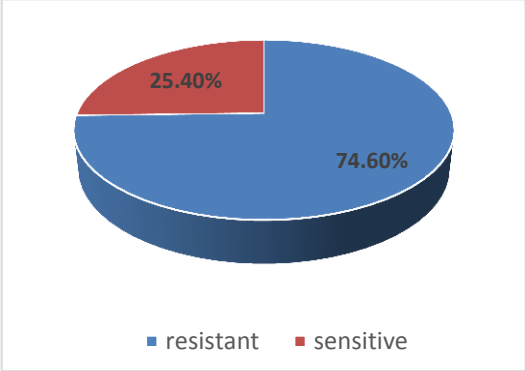

Fig. 1: Colistin resistant and sensitive *Enterobacteriaceae* in this study

Molecular detection of the gene *mcr-1:*

By employing conventional PCR, the *mcr-1* gene was found in 4 (7.4%) of the isolates, all of which were recognized as *E. coli*.

Table 2; demonstrates that samples that tested positive for the *mcr-1* gene had significantly greater levels of colistin MICs than isolates of *Enterobacteriaceae* which tested negative for the *mcr-1* gene. (P = 0.022).

Table 2: Correlation between *mcr-1* gene and colistin MIC

	Colistin MIC (µg/ml)			*P* value
	Median	Mini.	Maxi.	
***mcr-1* Positive**	8.00	4.00	16.00	0.022
***mcr-1* Negative**	4.00	1.00	32.00	

Effect of DPA addition on MIC:

When DPA was added, all strains carrying the *mcr-1* exhibited a decrease in the MIC of colistin by a minimum eightfold dilution (sensitivity 100%). However, 51% of the *mcr-1* negative strains showed a similar reduction of MIC (specificity 48.98%). The test demonstrated a positive predictive value of 13.79% and a negative predictive value of 100%. The DPA based assay presented accuracy of 52.83%. Summary of BMD results before and after addition of DPA is demonstrated in table 3.

Table 3: BMD results before and after DPA addition:

Colistin resistance mechanism	Number of isolates	MIC (µg/mL)	MIC after DPA addition	fold reduction in MIC
***mcr-1* positive**	4	4 to 8	≤0.125- 1	≥8
***mcr-1* negative**	25	4 to ≥32	≤0.125 – 4	≥8
***mcr-1* negative**	24	4 to ≥32	2 to 16	unchanged or <2

DISCUSSION

The detection of an effective colistin diagnostic is vital and potentially life-saving due to the significance of colistin in treating lethal infections brought on by Gram-negative bacteria which are drug resistant. Nevertheless, developing a diagnostic tool that is simple to incorporate into the routine of conventional microbiology laboratories continues to be a difficulty due to the labor-intensive nature of the recommended method for colistin sensitivity testing, the BMD. Furthermore, the easier to perform disc diffusion method and E-test contain intrinsic errors because of the difficulties in colistin diffusion through agar [13].

In our study, considering BMD method as the gold standard for detecting colistin resistance, it was revealed that out of 71 *Enterobacteriaceae* isolates studied, 53 (74.6%) were colistin resistant, while 18 (25.4%) were colistin sensitive. The resistance rate was 66.7% (32/48) among *E. coli* and 91.3% (21/23) among *Klebsiella pneumoniae*. These results were slightly higher than results reported by Jayol et al. [14] in 2019, who determined that out of 123 studied *Enterobacteriaceae* isolates (including 46 *K. pneumoniae* and 32 *E. coli* isolates), 67.5 % (83/123) were colistin-resistant and 32.5% (40/123) were colistin-susceptible with 53.1% (17/32) and 76% (35/46) resistance rate among studied *E. coli* and *K. pneumoniae* isolates respectively. A study by Elfeky et al. [15] in 2023 also stated that *K. pneumoniae* represented the majority (97.6%) of colistin-resistant *Enterobacteriaceae* isolates. Another study in Gaza reported that from 100 bacterial isolates, 41% were resistant to colistin, while 59% were sensitive [16].

The disparity of results in different studies might be due to the difference in the sample types, sample size, geographical regions.

The elevated colistin resistance observed in our study can be linked to the origin of the isolates from hospitalized patients with urinary tract infections. Resistance patterns tend to be more common among hospitalized patients, likely as a result of extended antibiotic usage and the utilization of urinary catheters

In the present study, we detected the *mcr-1* gene in 4 *E. coli* isolates that were resistant to colistin, accounting for 7.4% (4/53) of all colistin-resistant isolates. This rate is similar to that identified in a previous study conducted by La et al. [17] in Singapore, where patients had a high frequency of *mcr-1* gene, i.e, 6.0% and 8.0% estimated by culturing stool and direct stool PCR, respectively predominantly in *E. coli*. A lower rate was detected in another study which did not report any *mcr-1* or *mcr-2* out of 27 colistin resistant isolates [18].

The remaining colistin resistant isolates may contain other genes or chromosomally mediated resistance as the reason for the absence of *mcr-1* gene detection. A study done by Elshaer et al. [19] stated that the emergence of colistin resistance in gram-negative bacteria can be caused by mutational or adaptive mechanisms. Mutations usually affect the outer membrane of Gram-negative bacteria, where colistin acts.

The ability of DPA-based tests to detect *mcr-1* producers was assessed in the current study. It was found that all the isolates harboring *mcr-1* exhibited a decrease in colistin MIC with a minimum of eight-fold dilution on adding DPA, which makes the test 100% sensitive. However, 51% of *mcr-1* negative isolates showed comparable results, which make it 48.98% specific.

In accordance with our study, previous studies stated that the DPA-based assay is a good test to detect *mcr-1* positive *E. coli* [1, 20, 21].

In contrast to our results, Büdel et al. [1] could detect 37 out of 44 *mcr-1* positive strains by DPA-based assay, which yielded a sensitivity of 84.1% and 100% specificity. Similarly, Wink et al. [21] reported a sensitivity of 93.6% and 95.7% specificity.

This differences in sensitivity and specificity may be a result of the different prevalence of resistance to colistin and *mcr-1* gene among *Enterobacteriaceae* isolates [22], in addition to several research constraints, such as the restricted sample size and the number of investigated species, which make it challenging to evaluate the DPA-based assay for all *Enterobacteriaceae*.

CONCLUSION

In conclusion, it is important to consider the limitations of the DPA- based microdilution test. While it may not provide the most accurate detection method of *mcr-1* producers within *Enterobacteriaceae*, it can still be used as a screening test to identify the presence of the gene. The high rate of colistin resistance really poses a significant challenge that cannot be ignored. Hence, further research is really needed to develop improved detection methods and effective strategies to combat this issue.

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