

## ORIGINAL ARTICLE

# Assessment of Colistin Susceptibility among Carbapenem-Resistant Clinical Isolates

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## ABSTRACT

**Key words:**

Colistin, CBDE, BMD, mcr, mCIM

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**Background:** Colistin is the last treatment option for infections caused by carbapenem resistant Gram-negative bacilli (CRGNB). The increasing spread of chromosomally encoded and plasmid-mediated colistin resistance made colistin susceptibility assessment a necessity. **Objectives:** Assessment of colistin susceptibility in CRGNB by broth micro dilution method (BMD), as the standard method and colistin broth disk elution method (CBDE), as a substitute procedure with genotypic determination of plasmid mediated colistin resistance (*mcr*) genes. **Methodology:** CRGNB were collected and identified by conventional methods. Testing carbapenemase production by modified Carbapenem Inactivation Method (mCIM) and colistin susceptibility (by BMD and CBDE) were done and results were interpreted regarding CLSI (2022) guidelines followed by genotypic detection of *mcr-1*, -2, -3, -4, and -5 genes by multiplex PCR. **Results:** 155 out of 308 GNB (50.3%) were carbapenem resistant. Among them, 129 (83.2%) isolates were carbapenemase positive by mCIM. Colistin susceptibility testing by BMD revealed 43 out of 155 CRGNB isolates (27.7%) were colistin resistant. Sensitivity, specificity, PPV, NPV and accuracy of CBDE were 99.09%, 93.33%, 97.32%, 97.67%, 97.42% respectively with almost perfect agreement with BMD. By PCR, only 3 CRGNB isolates (6.98%) carried *mcr-1* while other *mcr* genes were not detected at all. **Conclusion:** Colistin resistance rate among CRGNB is concerning, causing serious and even deadly infections so prospective surveillance is essential. Broth disk elution method is a simple, non-expensive reliable option to test colistin susceptibility.

## INTRODUCTION

Antimicrobial resistance (AMR) remains a formidable and alarming public health threat worldwide. It causes high morbidity and mortality<sup>1</sup>. The capability of Gram-negative microbes to acquire mobile genetic elements including carbapenemase genes can confer augmented resistance limiting treatment options. These pathogens are included in both types of human infections, hospital and community with frequently express resistance to most antibiotics classes<sup>2</sup>. Thus, the determination of carbapenemase-producing organisms is paramount for treatment decisions beside infection control<sup>3</sup>.

The polymyxins including colistin are considered the last choice of antibiotics for treatment of infections with carbapenem resistant Gram-negative bacilli (CRGNB). Colistin interacts with the bacterial outer membrane by removing divalent cations from the negatively-charged phosphate groups of the Lipid A leading to cell lysis<sup>4</sup>. Colistin is very effective against most *Enterobacterales* and non-fermenting Gram-negative pathogens such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. On the contrary, colistin is inactive against Gram-negative cocci, anaerobic and Gram-positive bacteria. Also, Some *Enterobacterales*

such as *Proteus mirabilis*, *Morganella morganii*, *Serratia marcescens*, and *Burkholderia* spp have colistin intrinsic resistance as a result of genes' constitutive expression (i.e. *eptB*) which leads to lipopolysaccharide (LPS) modification<sup>5</sup>.

Bacteria have gained several mechanisms to protect themselves against colistin. The main mechanism is LPS modification through acquiring genes like mobile colistin resistance (*mcr*) and *PhoPQ* and *PmrAB* genes and its regulators genes (i.e. *mgrB*)<sup>6</sup>. In addition, bacteria can inactivate the biosynthesis of lipid A genes (*lpxA*, *lpxC* and *lpxD*) then, LPS will be completely lost. Other mechanisms of colistin resistance include efflux-pump systems overexpression and capsular polysaccharide overproduction<sup>5</sup>.

Recently, worldwide hospital outbreaks have occurred due to colistin-resistant strains. Acquired colistin resistance is based mainly on diverse chromosomal mutations, but the excessive usage of colistin in both veterinary and human health sectors has promoted development and spread of the plasmid-encoded mobile colistin resistance (*mcr*) genes (*mcr-1* to *mcr-10*) with several variants<sup>7</sup>.

Assessing colistin susceptibility remains a challenge. Large colistin molecular size in addition to its cationic nature resulting in poor colistin disk diffusion and

therefore, disk diffusion method was not recommended<sup>8</sup>. Broth micro dilution (BMD) was recommended as the standard procedure. This standard technique is quite laborious, time-consuming, difficult to interpret, susceptible to mistakes, not suitable for most laboratories<sup>8,9</sup>. Currently, agar dilution and colistin broth disk elution (CBDE) are acceptable methods<sup>10</sup>.

This study aimed to assess colistin susceptibility pattern among CRGNB by broth micro dilution method (BMD), as the standard method and colistin broth disk elution method (CBDE), as a substitute procedure with genotypic detection of plasmid mediated colistin resistance *mcr* genes (*mcr*-1, -2, -3, -4, and -5 genes).

## METHODOLOGY

### Study design and patients:

This comparative, cross-sectional study was conducted at Medical Microbiology Department and Molecular Unit, Central Laboratory, Faculty of Medicine, Menoufia University, during the period from November 2020 to March 2022. Clinical isolates (n=155 CRGNB) were obtained from Menoufia University Hospitals admitted patients (n= 674). Written informed consents were obtained from those patients and from the guardians of unaware patients upon sample collection. The study has been approved by the ethical committee, Faculty of Medicine, Menoufia University. Calculation of sample size was done using open Epi program with power of study 80% and confidence level 95%.

### Inclusion criteria

- Both genders (male and female).
- All age groups
- Patients showing signs of sepsis or pyogenic infections
- Carbapenem resistant GNB.

### Exclusion criteria:

- Patients refusing participation.
- Patients showed good response to antibiotic therapy.
- Culture did not meet the criteria of infection
- Pathogens having colistin intrinsic resistance e.g. *Proteus*, *Serratia* and *Morganella*

### Specimens:

Different microbial specimens were collected under aseptic conditions on clinical suspicion of infection according to standard definitions. Specimens included respiratory specimens, urine, blood, pus and wound swab specimens. Then specimens transported in suitable

transport media (when needed) to be processed in the Microbiology Laboratory.

### Bacterial identification and testing for antibiotic susceptibility

Collected specimens were cultured on nutrient agar, blood agar, and MacConkey agar plates (Oxoid, UK) then incubated aerobically at 37°C for 24 hours. Followed by identification of obtained colonies up to species level via conventional techniques<sup>11,12</sup>. Testing for Antimicrobial susceptibility was done using disk diffusion method against different antimicrobial agents (Oxoid, Basingstoke, UK) as recommended by Clinical Laboratory Standard Institute (CLSI)<sup>13</sup>. *Escherichia coli* ATCC 25922 had been used as standard quality control strain. Isolated Gram negative bacilli were further preserved on tryptic soy broth with 16% glycerol and frozen at -80°C

### Phenotypic detection of carbapenem resistance and carbapenemase production

Isolated Gram negative pathogens showing resistance to at least one of four carbapenems (imipenem, meropenem, doripenem and ertapenem) were further phenotypically screened for carbapenemase production by modified carbapenem inactivation method (mCIM) and EDTA-modified carbapenem inactivation method (eCIM)<sup>14</sup>.

### Phenotypic detection of colistin resistance:

Colistin minimum inhibitory concentration (MIC) for all CRGNB was determined by BMD method and CBDE methods. Regarding CLSI recommendations, Colistin MIC of  $\leq 2 \mu\text{g}/\text{mL}$  was considered intermediate, whereas MIC of  $\geq 4 \mu\text{g}/\text{mL}$  was considered resistant<sup>8</sup>.

### Genotypic detection of colistin resistance *mcr* genes:

The isolate that exhibited colistin MIC value  $\geq 4 \mu\text{g}/\text{mL}$  was further investigated for the existence of *mcr*-1, -2, -3, -4, and -5 genes. Bacterial DNA extraction and purification was performed using QIAamp DNA Mini Kit (Qiagen, Germany, cat. no. 51306). The used primers are listed in **table 1**. The amplification cycle was: 15 min at 94°C, followed by 25 cycles of 30 s at 94°C, 90s at 58 °C, 1 min at 72°C, and a final extension time of 10 min at 72°C. Electrophoresis was done with gel 2% for 20 minutes then the products were visualized by UV and compared with DNA ladder<sup>15</sup>.

### Statistical analysis:

Data coding, validation and analysis were conducted by the Statistical Package for the Social Sciences (SPSS), version 22 (SPSS Inc., Chicago, IL, USA). Frequencies and proportions were used to present the data.

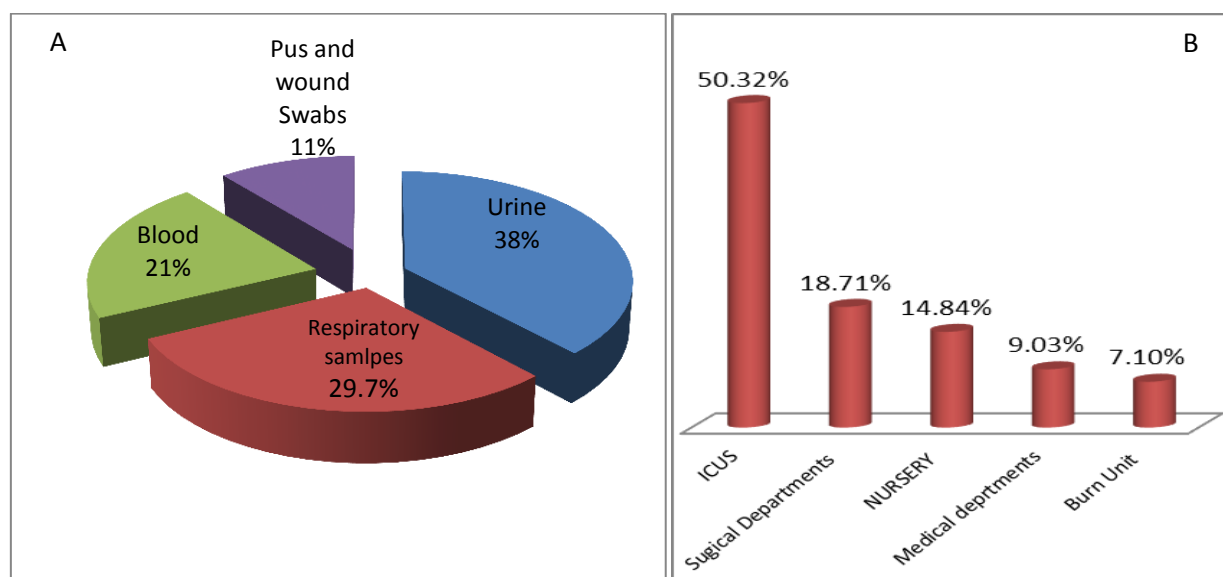
**Table 1: Primers sequences used for *mcr* gene detection**

Target gene	GenBank	Primers and conditions	Melting temperature - T <sub>m</sub> (°C)	Product size (bp)	Reference
<i>mcr-1</i>	KP347127	F 5'-AGTCCGTTTGTTCCTTGTGGC-3' R 5'-AGATCCTTGGTCTCGGCTTG-3'	58	320	15
<i>mcr-2</i>	LT598652	F 5'-CAAGTGTGTTGGTCGCAGTT-3' R 5'-TCTAGCCCAGACAAGCATACC-3'	58	715	15
<i>mcr-3</i>	KY924928	F 5'-AAATAAAAATTGTTCCGCTTATG-3' R 5'-AATGGAGATCCCCGTTTTT-3'	58	929	15
<i>mcr-4</i>	MF543359	F 5'-TCACTTTCATCACTGCGTTG-3' R 5'-TTGGTCCATGACTACCAATG-3'	58	1116	15
<i>mcr-5</i>	KY807921	F 5'-ATGCGGTTGTCTGCATTATC-3' R 5'-TCATTGTGGTTGTCCTTTTCTG-3'	58	1644	15

## RESULTS

Overall, 308 no duplicate clinical GNB pathogens were isolated, including 114 *E. coli* (37%), 96 *Klebsiella spp* (31.2%), 42 *Pseudomonas aeruginosa* (13.6%), 23 *Acinetobacter spp* (7.5%), 20 *Enterobacter spp* (6.5%) and 13 *Citrobacter spp* (4.2%) retrieved from 187 male (60.7%) and 121 female (39.3%) patients from all age groups (mean =44±27 years) admitted to different Departments of Menoufia University Hospitals from November 2020 to March 2022.

Susceptibility testing revealed 155 carbapenem resistant isolates out of 308 GNB (50.3%). The most frequent carbapenem resistant pathogen was *Klebsiella spp* (56/155, 36.1%) followed by *Escherichia coli* (51/155, 32.9%), *Pseudomonas aeruginosa* (20/155, 12.9%), *Acinetobacter spp* (13/155, 8.4%), *Enterobacter spp* (9/155, 5.8%) and *Citrobacter spp* (6/155, 3.9%). The sources of carbapenem resistant isolates were urine (38.1%), respiratory samples (29.7%), blood (21.3%), pus and wound swabs (10.9%). Intensive Care Units were the main source of these samples (50.32%), followed by surgical departments (18.71%), nursery (14.84%), medical departments (9.03%) and burn unit (7.1%) as illustrated in figure 1.

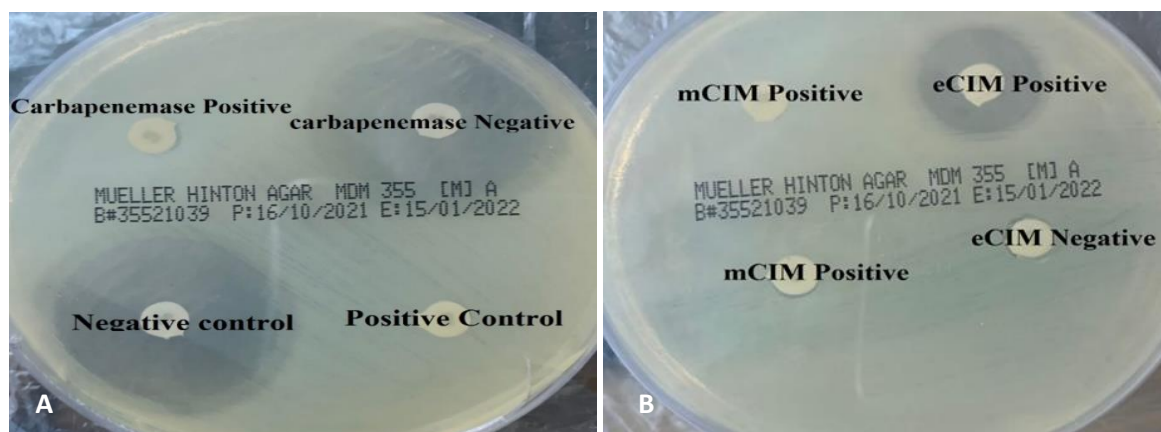


**Fig. 1:** Source of carbapenem resistant isolates. (A) Clinical specimens; (B) Departments

The highest antimicrobial susceptibility pattern among CRGNB isolates was to colistin (72.3%), followed by ceftazidime-avibactam (67.7%), and tigecycline (63.2%). While the highest antimicrobial resistance was observed against ampicillin and aztreonam (100% for each), followed by ampicillin-sulbactam, amoxicillin-clavulanate (98% for each), cefuroxime (96.8%), ceftriaxone (95.5%), cefotaxime, ceftazidime (94.9%), cefepime (87%), ciprofloxacin

(86.5%), piperacillin-tazobactam (84.5%), gentamicin (82.6%), levofloxacin (81.3%), trimethoprim-sulfamethoxazole (76.2%) and amikacin (73.5%).

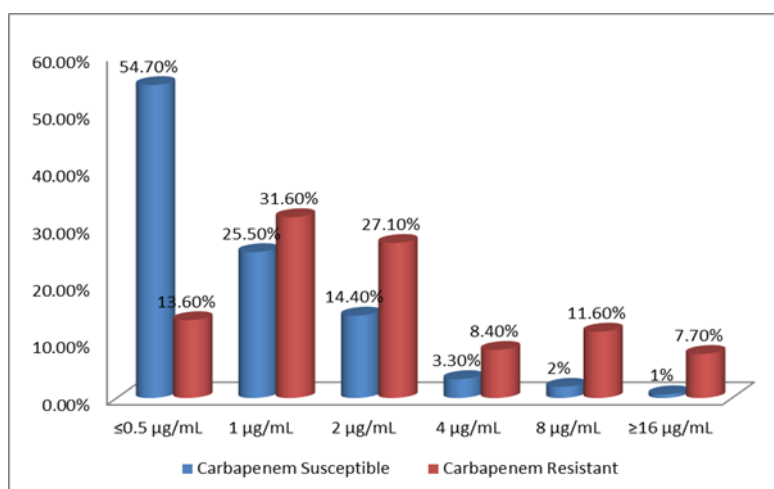
In this study, carbapenemase production was the main carbapenem resistance mechanism. As we detected 129 out of 155 CRGNB isolates (83.2%) were carbapenemase positive by mCIM among them 85 isolates (65.9%) were metallo-β lactamase producers by eCIM (figure 2).



**Fig. 2:** Phenotypic detection of carbapenemase production. (A) Modified Carbapenem Inactivation Method (mCIM); (B) enhanced Carbapenem Inactivation Method (eCIM)

Regarding colistin MIC by BMD, CRGNB isolates demonstrated the maximum colistin MIC at 2 µg/mL (31.6%), followed by 1 µg/mL (27.1%), ≤0.5 µg/mL (13.6%), 8 µg/mL (11.6%), 4 µg/mL (8.4%) and ≥16 µg/mL (7.7%). While, carbapenem susceptible GNB isolates demonstrated the maximum colistin MIC at ≤0.5 µg/mL (54.2%) followed by 1 µg/mL (25.5%), 2 µg/mL (14.4%), 4 µg/mL (3.3%), 8 µg/mL (01.95%) and ≥16 µg/mL (0.65%) as illustrated in figure 3 and

table 2. There was a high statistically significant difference ( $p > 0.001$ ) between carbapenem susceptible and carbapenem resistant GNB isolates regarding colistin susceptibility. As 43/155 (27.7%) of carbapenem resistant GNB isolates were colistin resistant. While, only 9/153 (5.9%) of carbapenem susceptible GNB isolates were colistin resistant as illustrated in table 3.



**Figure 3:** Colistin MICs by BMD among studied GNB isolates

**Table 2: Distributions of colistin MICs determined by BMD among studied GNB isolates**

Pathogen	Carbapenem susceptibility	Colistin MIC by BMD					
		Colistin susceptible			Colistin resistant		
		≤0.5 µg/mL	1 µg/mL	2 µg/mL	4 µg/mL	8 µg/mL	≥16 µg/mL
<i>E. coli</i> (n=114)	S (n= 63)	39(62%)	17(27%)	5(7.9%)	2(3.1%)	0(0%)	0(0%)
	R (n= 51)	7(13.7%)	21(41.2%)	10(19.6%)	5(9.8%)	6(11.8%)	2(3.9%)
<i>Klebsiella</i> spp (n= 96)	S (n=40)	21(52.5%)	9(22.5%)	7(17.5%)	3(7.5%)	0(0%)	0(0%)
	R (n=56)	2(3.6%)	18(32.1%)	19(33.9%)	6(10.7%)	8(14.3%)	3(5.4%)
<i>Pseudomonas aeruginosa</i> (n= 42)	S (n=22)	13(59.1%)	7(31.8%)	1(4.55%)	0(0%)	1(4.55%)	0(0%)
	R (n= 20)	7(35%)	3(15%)	4(20%)	1(5%)	3(15%)	2(10%)
<i>Acinetobacter</i> spp (n= 23)	S (n=10)	4(40%)	3(30%)	2(20%)	0(0%)	1(10%)	0(0%)
	R (n= 13)	3(23.1%)	1(7.7%)	5(38.4%)	1(7.7%)	0(0%)	3(23.1%)
<i>Enterobacter</i> spp (n=20)	S (n=11)	5(45.4%)	1(9.1%)	3(27.3%)	0(0%)	1(25%)	1(9.1%)
	R (n= 9)	1(11.11%)	3(33.33%)	2(22.22%)	0(0%)	1(11.11%)	2(22.22%)
<i>Citrobacter</i> spp (n= 13)	S (n=7)	1(14.3%)	2(28.6%)	4(57.1%)	0(0%)	0(0%)	0(0%)
	R (n= 6)	1(16.7%)	3(50%)	2(33.3%)	0(0%)	0(0%)	0(0%)
Total (n=308)	S (n= 153)	83(54.2%)	39(25.5%)	22(14.4%)	8(5.2%)	1(0.7%)	0(0%)
	R (n= 155)	21(13.6%)	49(31.6%)	42(27.1%)	13(8.4%)	18(11.6%)	12(7.7%)
	TOTAL (n=308)	104(33.8%)	88(28.6%)	64(20.8%)	18(5.8%)	21(6.8%)	13(4.2%)

**Table 3: Relation between colistin and carbapenem susceptibility patterns**

Carbapenem susceptibility	Colistin susceptibility		Chi square	P value
	Susceptible	Resistant		
Susceptible (n=153)	144 (94.1%)	9 (5.9%)	26.22	<0.001
Resistant (n=155)	112 (72.3%)	43 (27.7%)		
Total (n=308)	256 (83.1%)	52 (16.9%)		

As colistin resistance was detected in 43 CRGNB isolates (27.7%) per the reference BMD method, the majority of isolates were *Klebsiella* spp (17/43, 39.5%) followed by *Escherichia coli* (13/43, 30.2%), *Pseudomonas aeruginosa* (6/43, 14%), *Acinetobacter* spp (4/43, 9.3%) and *Enterobacter* spp (3/43, 7%). While all carbapenem resistant *Citrobacter* spp isolates were colistin susceptible.

Considering BMD, as the standard colistin susceptibility testing method. The sensitivity,

specificity, PPV, NPV and accuracy of CBDE were 99.09%, 93.33%, 97.32% 97.67%, 97.42% respectively. With almost perfect agreement between the two methods in detection of colistin susceptibility (Table 4).

By PCR, *mcr-1* gene was detected in 3 out of 43 colistin-resistant CRGNB isolates (6.98%). The three isolates were *E. coli*. While *mcr-2*, -3, -4, and -5 were not detected at all in any of our tested isolates.

**Table 4: Performance features of CBDE in relation to BMD**

Colistin susceptibility by CBDE	Colistin susceptibility by BMD		
	Susceptible (n=110)	Resistant (n=45)	Total (n=155)
	No (%)	No (%)	No (%)
Susceptible	109 (99.1%)	3 (6.7%)	112 (72.3%)
Resistant	1 (0.9%)	42 (93.3%)	43 (27.7%)
Total	110	45	155
Kappa	0.93*		
Sensitivity	99.09%		
Specificity	93.33%		
PPV	97.32%		
NPV	97.67%		
Accuracy	97.42%		

\* Kappa between 0.81 and 1.00: almost perfect agreement



## DISCUSSION

Infections with CRGNB are increasing globally, limiting the available therapeutic options for their treatment. Colistin is recommended as one of the last-resorts to treat these infections. Therefore, it is crucial to monitor its susceptibility<sup>16</sup>.

In this study, 155 out of 308 GNB isolates (50.3%) were carbapenem resistant. *Klebsiella* spp were the most frequent CRGNB isolates (36.1%) followed by *Escherichia coli* (32.9%), *Pseudomonas aeruginosa* (12.9%), *Acinetobacter* spp (8.4%), *Enterobacter* spp (5.8%) and *Citrobacter* spp (3.9%). Matching with carbapenem resistance rates (54.5% and 55.8%) in previous studies<sup>17,4</sup>. On the contrary, lower rates (18.5% and 23.1%) were reported in other studies<sup>18-19</sup>. *K. pneumonia* was the most common isolated CRGNB in some studies<sup>20-21</sup> but *E.coli* was the commonest (56.5% and 50%) in others<sup>17, 22</sup>.

In our study, sources of CRGNB isolates were urine (38.1%), followed by respiratory samples (29.7%), blood (21.3%), and pus and wound swabs (10.9%). Intensive Care Units were the main source of these samples (50.32%), followed by surgical departments (18.71%), nursery (14.84%), medical departments (9.03%) and burn unit (7.1%). Kra et al<sup>17</sup> reported the same observations. While, Saed et al mentioned that tracheal aspirate was the main source of CRGNB isolates<sup>20</sup>.

Carbapenem resistant *Enterobacteriaceae* (CRE) comprise both carbapenemase producing (CP-CRE) which produce carbapenemase enzymes hydrolyzing carbapenem and non-carbapenemase producing CRE (non-CP-CRE) strains that have other causes like drug efflux pumps and outer membrane protein structural mutations resulting in drug impermeability<sup>4,23</sup>. In this research, 129 of CRGNB isolates (83.2%) were carbapenemase positive by mCIM, among them 85 isolates (65.9%) were metallo- $\beta$  lactamase producers. This finding goes in line with results of Laolerd et al<sup>24</sup>, Khattab et al<sup>4</sup> and Qadri et al<sup>25</sup> which observed that carbapenemase production was the main carbapenem resistance mechanism with 77.7%, 75.6% and 67.3% carbapenemase production rates respectively. But decreased rate (18.5%) was detected by Kandeel<sup>26</sup>. While, Ngbede et al observed that none of their isolated carbapenem resistant organisms was carbapenemase producer nor harbor any known carbapenemase producing genes<sup>18</sup>.

Regarding colistin MIC by BMD, our findings revealed that 5.7% of carbapenem susceptible and 27.7% of CRGNB isolates were non susceptible to colistin with a high statistically significant difference ( $p < 0.001$ ). Approximately similar colistin susceptibility pattern was detected<sup>17</sup>. However higher rates (64.8% and 53%) were detected in France<sup>27</sup> and in Egypt<sup>9</sup> respectively, lower rates (16.4% and 2.79%)

were reported in previous Egyptian studies<sup>21,28</sup>. The difference in colistin resistance prevalence rate in previous studies resulting from the diversity in geographical regions, study populations, studied cases numbers, the patients' general condition, type of collected samples, adherence to the infection control procedures, implementation of antibiotic stewardship programs and used antibiotic.

In our research, the most common colistin resistant CRGNB isolate was *Klebsiella* spp (39.5%). Similar finding was reported<sup>21, 29</sup>. But, *Enterobacter* was the commonest colistin-resistant strain in Prim et al study.<sup>30</sup>

Our findings revealed that, sensitivity, specificity, PPV, NPV and accuracy of CBDE were 99.09%, 93.33%, 97.32% 97.67% and 97.42% respectively with almost perfect agreement in correlation to BMD. Matching with F'oldes et al who reported that sensitivity, specificity, PPV, NPV and accuracy of CBDE were 100% (for each) with total agreement correlated to BMD<sup>2</sup>. Colistin broth disk elution method is an alternative simple, easy, non-expensive reliable option for assessing colistin sensitivity.

Among colistin-resistant isolates (n=43), we found *mcr-1* gene in only 3 (6.98%) *E. coli* isolates. Similar *mcr-1* prevalence rates (7.5% and 8.1%) were mentioned in Egypt<sup>9</sup> and in Hong Kong<sup>31</sup> respectively. Although, lower rate (2.9%) was reported in Egypt<sup>28</sup>, higher rates were reported in North Italy (28.9%)<sup>32</sup> and South Africa (83%)<sup>33</sup>. A higher existence rate of the *mcr-1* gene in animal origin (33.3%) was reported<sup>34</sup>. Colistin overuse and abuse of in the poultry industry and agriculture may be the key factor of *mcr-1* high prevalence rate in bacteria isolated from animals and their products which may be the potential sources of *mcr-1* in human's origin. Plasmid mediated *mcr-2*, -3, -4, and -5 genes were not determined at all in any of our tested isolates. The same findings were observed by Anan et al.<sup>8</sup>

## CONCLUSION

The high colistin resistance prevalence has highlighted the possibility of losing colistin efficiency against CRGNB. Prospective surveillance should be implemented. Colistin broth disk elution method is a simple, easy, non-expensive reliable option for testing colistin susceptibility. Efforts should be made to inhibit the emergency of CRGNB through the wise use of antimicrobial agents and strict adherence to infection control procedures including: implementation of CRE initial screening policy, patient isolation, initiation of contact precautions in addition to standard precautions, strengthening of environmental cleaning and disinfection with monitoring of cleaning performance through checklist, continuous surveillance,

implementation of antibiotic stewardship program, antibiogram and MDRO care bundles.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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