Colistin susceptibility among multidrug resistant Gram-negative bacilli isolated from Tertiary hospital in Egypt

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Abstract

Colistin is considered as the last resort for treatment of bacterial infections caused by carbapenem-resistant Gram-negative bacilli (GNB). Colistin resistance can increase due to the spread of plasmid-borne *mcr-1* gene. This study aimed to determine colistin susceptibility and to detect the presence of *mcr-1* gene in the clinical isolates of GNB recovered from different clinical samples collected from Ain Shams University Hospital, Cairo, Egypt. About thirty-five GNB isolates were recovered from the different clinical samples that were collected during the period from February-April, 2019. These isolates were subjected to antibiotic susceptibility testing using disc diffusion assay, and colistin susceptibility through the E-test. In addition, conventional Polymerase chain reaction (PCR) was carried out for detection of *mcr-1* gene among the colistin GNB resistant isolates. Most of the GNB isolates (60 %) were recovered from blood samples. *Klebsiella pneumoniae* (*K. pneumoniae*) was the most common isolated bacterium; that was represented by 24 isolates (68%). Out of the 35 GNB, only five isolates (14.3 %) were resistant to colistin by E-test, with MIC >256 μg/ ml. The *mcr-1* gene was detected only in one *Pseudomonas aeruginosa* (*P. aeruginosa*) isolate. This study concluded the low frequency of *mcr-1* gene among the current GNB isolates. However, a large scale study is recommended to detect colistin resistance among GNB.

Keywords: Colistin susceptibility, *mcr-1* gene, Multi-drug resistant GNB, PCR

1. Introduction

Colistin, was first recognized as a narrow spectrum antibiotic with potent activity against multi-drug resistant (MDR) Gram-negative bacteria (GNB) in the 1940s, but its use as systemic antibiotic was reduced as being nephrotoxic and neurotoxic (Falagas *et al*., 2005). According to the study conducted by Yu *et al*., (2015), colistin interacts with the lipid A of lipopolysaccharides (LPSs) on the outer membrane of

968

GNB and displaces the calcium and magnesium bridges that stabilize the LPS. Subsequently, colistin permeabilizes the bacterial outer membrane and disrupts the integrity of the inner membrane, ultimately causing cell death. The emergence of resistance against colistin has been detected in different countries worldwide in recent years (Poirel *et al*., 2017). There are two mechanisms of colistin resistance; either plasmid acquiring resistance or chromosomal mutations (Liu *et al*., 2016). A previous study conducted by Baron *et al*., (2016) reported that the chromosomally-mediated or intrinsic colistin resistance is mediated by different complex mechanisms that lead to the loss or modification in the production of lipopolysaccharide in GNB, and this type of resistance may develop during treatment by colistin drug. On the other hand, the plasmid-mediated colistin resistance is due to *mcr-1* gene that was discovered in southern China in 2015, and was recorded in 20% of animal strains and 1% of human strains (Liu *et al*., 2016). Recently, Dalmolin *et al*., (2018) revealed the detection of *mcr-1* gene among MDR Gram-negative species in different countries since its discovery. In Egypt, *mcr-1* gene was firstly detected in an *E. coli* isolate in 2016 from patient with bacteremia (Elnahriry *et al*., 2016).

Olaitan *et al*., (2014) highlighted that colistin resistance mediated by this gene is a stable resistance not related to the use of colistin, and is found essentially in *E. coli*, *P. aeruginosa* and *K. pneumoniae*. Later, Poirel *et al*., (2017) added that transmission of the resistant bacteria in the hospital settings is another way by which the prevalence of colistin resistance can increase, due to spread of the plasmid-borne *mcr-1* mediated resistance. The objectives of the present study were to determine the colistin susceptibility, and detect the presence of *mcr-1* gene in clinical isolates of GNB isolated from different clinical samples from Ain Shams University Hospital.

2. Material and methods

2.1. Bacterial isolates

This study was conducted on 35 clinical isolates of GNB isolated from different clinical samples from patients admitted to the Intensive care unit (ICU) of Ain Shams University Hospital, during the period from February to April, 2019. The collected samples included; blood, urine, sputum, endotracheal aspirate and wound swabs. All the bacterial isolates were identified by conventional bacteriological methods according to Collee *et al*., (1996), based on colonial morphology, microscopic examinations of Gram stained films and several biochemical assays.

2.2. Antibiotics susceptibility testing

All the bacterial isolates were tested for antibiotics susceptibility testing using Kirbey-Bauer disc diffusion method on Muller-Hinton agar plates, and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI. 2019) guidelines. The tested antibiotics (supplied by Oxoid, Basingstoke, UK) included; Amoxicillin/ Clavulanic acid (AMC, 20/10 μg), Piperacillin/ tazobactam (TPZ, 100/10 μg), Ceftriaxone (CRO, 30 μg), Ceftazidime (CAZ, 30 μg), Cefotaxime (CTX,30 μg), Cefotaxime/ calvulenic acid (30/10 μg), (CTX-Clav, 30/10 μg), Ceftazidime/ calvulenic acid (CAZ-Clav ,30/10 μg) ,Cefoxitin (FOX, 30 μg), Cefoperazone (CEP, 30 μg), Cefoperazone/ sulbactam (CES, 105 μg), Cefepime (FEP, 30 μg), Imipenem (IPM, 10 μg), Meropenem (MEM, 10 μg), Gentamicin (CN, 10 μg), Amikacin (AK, 30 μg), Ciprofloxacin (CIP, 5 μg), Levofloxacin (LEV, 5 μg) and Colistin (CT, 10 μg). A clinical strain of *E. coli* ATCC 25922 was supplied by the Central Health Laboratories, Ministry of Health and Population, Egypt, served as a quality control for the antibacterial susceptibility testing. The tested bacterial isolate is considered MDR if it was resistant to three or more classes of these antibacterial agents, according to Magiorakos *et al*., (2012).

2.3. Determination of colistin minimum inhibitory concentration (MIC)

Colistin MIC was determined with the E-test (BioMérieux, Marcy l'Etoile, France), according to the

manufacturer's recommendations. MICs were evaluated and interpreted based on the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST. 2017). A density of a 0.5 McFarland standard bacterial suspension of each isolate was swabbed onto the surface of Mueller-Hinton agar plates. E-test colistin strip (ranging from 0.16- 256 μg/ ml) was applied separately on the surface of each seeded plate using a sterile forceps, and then the plates were incubated at 35°C for 16 to 20 h.

2.4. Molecular detection of colistin resistance gene (*mcr-1*)

All colistin resistant isolates detected by the E-test were tested using conventional PCR for harboring Colistin resistance gene *mcr-1*. DNA extraction was carried out using QIAGEN DNA extraction Kit® (QIAGEN, USA), and purification of DNA from the bacterial isolates were done using the spin column method as per manufacturer's instructions.

Amplification of *mcr-1* gene of the isolates was carried out using a primer of 309 bp supplied from Invitrogen (F: 5/-CGGTCAGTCCGTTTGTTC-/3, R:5/-CTTGGTCGGTCTGTA GGG-/3), according to Liu *et al*. (2016). PCR products were run on 1.5% agarose gel, stained with ethidium bromide, finally visualized under UV light and then photographed.

2.5. Statistical analysis

All data were analyzed using SPSS for Windows version 22.0 (IBM Corp. 2013). Descriptive statistics, frequencies and percentages were calculated.

3. Results

3.1. Bacterial isolates identification

Klebsiella pneumoniae is the most common isolated bacteria, it is represented by 24 isolates (68 %), followed by *E. coli* (8, 23 %) and both are mainly isolated from blood samples (60 %), *P. aeruginosa* (2, 6%) and *Acinetobacter* sp. (1, 3%) as shown in Table (1).

Table 1: Frequency of isolated GNB and sample distribution

Samples (no.)	*K. pneumoniae*	*E. coli*	*P. aeruginosa*	*Acintobacter* sp.
Urine (3)	1(4.2%)	2 (25%)		
Blood (21)	15 (62.5%)	5 (62.5)	1(50%)	
Sputum (2)	1 (4.2%)		1(50%)	
Endotracheal aspirate (8)	6 (25%)	1(12.5%)		1(100%)
Wound (1)	1(4.2%)			
Total = 35	24 (68.6%)	8 (22.8%)	2 (5.7%)	1 (2.9%)

3.2. Antibiotics sensitivity pattern of the isolated bacterial spp.

Almost all the GNB isolates (100 %) are resistant to the beta-lactam/beta-lactamase inhibitor

combinations; 3rd generation cephalosporine, cefepime (FEP) and gentamicin (CN). About 33 isolates (94.2 %) are resistant to ciprofloxacin (CIP), 32 isolates (91.4%) are resistant to amikacin (AK) and levofloxacin (LEV), and 31 isolates (88.5%) are

970

resistant to imipenem (IPM) and meropenem (MEM). Colistin (CT) is the most sensitive antibiotic; where 6 bacterial isolates (17.1 %) are sensitive to it, as demonstrated in Table (2).

Table 2: Antibiotics sensitivity pattern of the recovered bacterial spp.

Name of antibiotic	Sensitivity no. (%)	Resistance no. (%)
AMC	0 (0%)	35 (100%)
TPZ	2 (5.8%)	33 (94.2%)
CRO	0 (0%)	35 (100%)
CAZ	0 (0%)	35 (100%)
CTX	0 (0%)	35 (100%)
CTX-Clav	0 (0%)	35 (100%)
CAZ-Clav	0 (0%)	35 (100%)
FOX	2 (5.8%)	33 (94.2%)
CFP	0 (0%)	35 (100%)
CES	0 (0%)	35 (100%)
FEP	0 (0%)	35 (100%)
AK	3 (8.6%)	32 (91.4%)
CN	0 (0%)	35 (100%)
LEV	3 (8.6%)	32 (91.4%)
CIP	2 (5.8%)	33 (94.2%)
MEM	4(11.5%)	31(88.5%)
IPM	4 (11.5%)	31(88.5%)
CT	6 (17.1%)	29 (82.9%)

Where; AMC (Amoxicillin/ Clavulanic acid), TPZ (Piperacillin/ tazobactam), CRO (Ceftriaxone), CAZ (Ceftazidime), CTX-Clav (Cefotaxime/ calvulenic acid), CAZ-Clav (Ceftazidime/ calvulenic acid), FOX (Cefoxitin), CEP (Cefoperazone), CES (Cefoperazone/ sulbactam), FEP (Cefepime), IPM (Imipenem), MEM (Meropenem), CN (Gentamicin), AK (Amikacin), CIP (Ciprofloxacin), LEV (Levofloxacin) and CT (Colistin)

3.3. Determination of MIC of colistin among GNB

Out of the 35 GNB isolates, only five isolates (14.3%) are resistant to colistin by the E-test with MIC > 256 µg/ ml. The MIC of colistin ranged from 0.125-256 µg/ ml as presented in Table (3), and Fig. 1(a, b).

Table 3: Colistin MIC of the bacterial isolates

Colistin (MIC)	No of isolates (%)
0.125 µg/ ml	1 (2.9%)
0.5 µg/ ml	2 (5.7%)
0.75 µg/ ml	12 (34.3%)
1 µg/ ml	14 (40%)
1.5 µg/ ml	1 (2.8)
>256 µg/ ml	5 (14.3%)

(a)   (b)

Fig. 1: (a): *Escherichia coli* isolate sensitive to colistin with MIC of 0.75 µg/ ml; (b): Resistance of *E. coli* isolate to colistin, with MIC >256 µg/ ml

3.4. Molecular detection of *mcr-1* gene in the colistin resistant GNB isolates

Out of the 5 colistin resistant isolates detected by the E-test, only single *P. aeruginosa* isolate is positive for *mcr-1* gene. The product amplified through PCR is

identified at 309 base pair (bp) by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, finally visualized under UV light using 100 bp DNA ladder, as clear in Fig. (2).
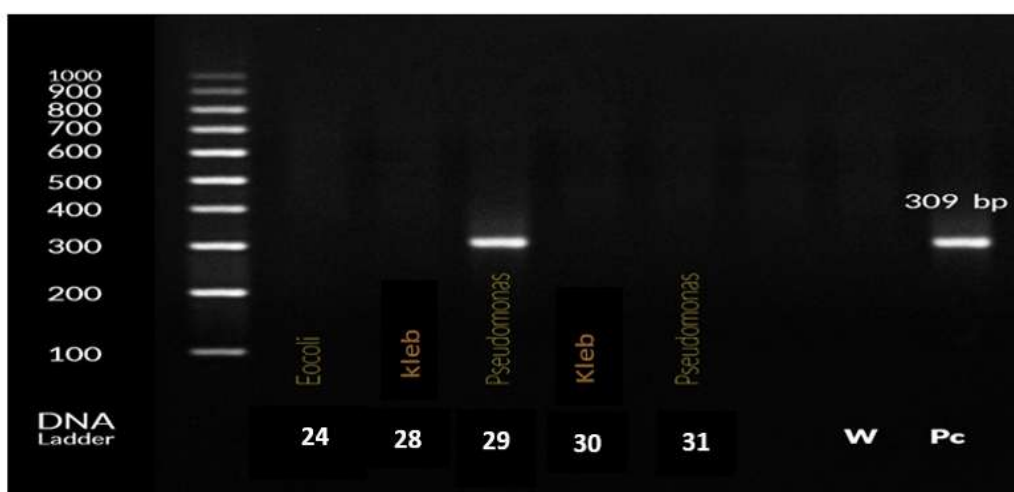
Fig. 2: Positive detection of *mcr-1* gene in *P. aeruginosa* isolate (no. 29) recording a single band of 309 bp

4. Discussion

Multidrug resistant Gram-negative bacteria (MDR-GNB) have become a major public health threat, as there are fewer or even sometimes no effective antimicrobial agents available (Mehrad *et al.,* 2015). According to See *et al*., (2013), a high rate of antimicrobial resistance has been reported in Egypt since more than 20 years, recorded among GNB causing nosocomial infections and outbreaks. In this study, all isolates (100%) were MDR as they were resistant to all beta-lactam/beta-lactamase inhibitor combinations; 3rd generation cephalosporine, cefepime and gentamicin. These results are in agreement with those of Allam *et al*., (2019), who found that all Enterobacteriaceae were 100% resistant to 7

antibiotics including; Ampicilln, Amoxicillin, Pinicillin, Piperacillin, Oxacillin, Amoxicillin/ Clavulanic acid and Ceftazidime, in their study that was conducted at Tanta Chest Disease Hospital, Egypt. High rates of carbapenem resistance were reported among the current isolates (88.5%), and this result was in accordance with Talaat *et al*., (2016); El-Kholy *et al*, (2020).

A recent study conducted by Newton-Foot *et al*., (2017) revealed that colistin is considered as the last resort of treatment for serious infections caused by carbapenem-resistant GNB, which are resistant to all other classes of antimicrobial agents. Thus, it is critical that clinical microbiology laboratories be able to identify colistin sensitivity among carbapenem

973

resistant GNB. According to the previous work of Falagas *et al*., (2005), most clinical microbiology laboratories rely on disc diffusion susceptibility testing method. In fact, several studies of Tan and Ng, (2006); Girardello *et al*., (2018) have found disc diffusion as an inherently unreliable susceptibility testing method to measure susceptibility of colistin. Tan and Ng, (2006) compared the performance of multiple colistin disk diffusion methods to that of the agar dilution method, and demonstrated that 79% and 89% of the colistin-resistant isolates were reported to be falsely susceptible by the disk diffusion methods. In this study, 68.6% (n= 24) of the colistin-resistant isolates tested by disc diffusion were susceptible when examined by the E-test. A recent study of Giske and Kahlmeter, (2018) reported that these false resistant results were attributed to the poor diffusion of the large cationic molecules of the colistin in agar, thus leads to problems in the standardization of sensitivity tests performed with this method.

Measuring colistin MIC by the broth micro-dilution is the most reliable method for determination of the colistin susceptibility, as recommended by the joint CLSI–EUCAST Polymyxin Breakpoints Working Group (EUCAST. 2016). However, its application is difficult to be done as a routine test.

The E-test is a simple and alternative method for the susceptibility testing of colistin. It has been validated by Tan and Ng, (2007) as a method with good concordance (> 96%) with agar dilution, and with micro-broth dilution methods by Behera *et al*., (2010). In this study, 5 isolates (14.3%) were recorded as resistant to colistin by E test with MIC> 256 μg/ ml. This result is in agreement with a recent study conducted by Shaban *et al,* (2020) at Ain Shams University Hospital, Cairo, Egypt. They recorded that four isolates (6.7%) were colistin resistant, with MICs ranging from 4 – 64 μg/ ml. Moreover, current result is in accordance with Zafer *et al*., (2019) who found that 36/450 (8%) of the isolates were colistin-resistant by E-tests during their study that conducted at the National Cancer Institute, Egypt. In addition, Rabie and Abdalla, (2020) also detected 24 isolates (12%)

out of 200 *E. coli* and *K. pneumoniae* isolates as colistin resistant using the tube micro-dilution method. Higher percentage of colistin resistance were also reported by El-Mokhtar *et al*., (2019), who recorded 10 (20.8%) and 12 (23.1%) *E. coli* strains as resistant to colistin, isolated from Assiut University Hospital and Minia University Hospital, Egypt, respectively.

Currently, the five colistin resistant isolates (14.3%) included *P. aeurginosa* (2) and *K. pneumoniae* (2) and *E. coli* (1) isolate . Similarly, Emara *et al*., (2019) detect that 10 isolates (16.4%) were resistant to colistin out of 61 GNB. These isolates were recovered from Tanta University Hospital, Egypt, including; *K. pneumoniae* (8), *E. coli* (1) isolate and *P. aeruginosa* (1) isolate. Higher percentages of colistin-resistant *E. coli* and *K. pneumoniae* were detected in a recent work conducted by Rabie and Abdalla, (2020), who found that 66.7% (n=16) of their isolates were *K. pneumoniae* and 33.3% (n=8) were *E. coli*. Different results were reported by Shaban *et al*., (2020), as they found *P. aeruginosa* (2/19, 10.5%) and *Acinetobacter baumannii* (2/14, 14.3%) were recorded as colistin resistant isolates.

In this study, the most common sources of colistin resistant isolates were from the respiratory specimens 2/5 isolates (40%), in accordance with Emara *et al*., (2019). Moreover, blood is also a common source of colistin resistant isolates in the current study, in agreement with Zafer *et al*., (2019). This result was against many other studies that reported urine as the most common source of colistin resistant isolates (Zaki *et al*., 2018; Rabie and Abdalla, 2020; Shaban *et al*., 2020). However, in our study only 1 /5 isolates was isolated from urine.

Colistin resistance mediated by plasmid encoded gene (*mcr-1*) is a dangerous problem as it can be easily transmitted between Gram negative bacteria. After *mcr1* gene discovery in China, many countries worldwide have reported its presence in Gram-negative bacteria (Malhotra-Kumar *et al*., 2016; Trung *et al*., 2017; Cyoia *et al*., 2019; Vounba *et al*., 2019). In Egypt, *mcr-1* gene was present in only one *E. coli*

isolate, which was recovered from the sputum of a patient with bacteremia who was hospitalized in the ICU of a Cairo City hospital, with no history of traveling abroad (Elnahriry *et al*., 2016). In the present study, the *mcr-1* gene was detected by conventional PCR in only one *P. aeurginosa* isolate out of five colistin resistant isolates. This result is in accordance with Shabban *et al*., (2020) who detected *mcr-1* gene in three of the phenotypically resistant isolates mainly; *P. aeruginosa* (1) and *A. baumannii* (2).

Several studies reported the detection of *mcr-1* among *E. coli* and *K. pneumoniae* isolates (Zaki *et al*., 2018; Moosavian and Emam, 2019; Rabie and Abdallah, 2020). Also, Liu *et al*., (2016), detected the *mcr-1* gene in 21 colistin resistant *E. coli* out of 40 (52.5%), and attributed their higher rates of *mcr-1* carriage to the high amount of livestock and meat in China, where prevalence of colistin-resistant isolates was high. However, in this study *mcr-1* gene wasn't detected among *E. coli* (1) and *K. pneumoniae* (2) colistin resistant isolates. This result is in agreement with Tanfous *et al*., (2018); Emara *et al*., (2019), who reported that colistin *mcr-1* gene was not detected among their phenotypically resistant isolates. This result could be attributed to the inability of PCR to predict colistin susceptibility as it doesn't exclude chromosomal colistin resistance, and there are new *mcr* genes responsible for colistin resistance that were not investigated in this study, according to WHO. (2018).

Conclusion

From results of the current study, we concluded the detection of high rates of MDR-GNB among the recovered clinical bacterial isolates, and the emergence of colistin resistance recorded through the E-test. In addition to the low frequency of *mcr-1* gene in the obtained isolates except for a single isolate of *P. aeruginosa*.

Conflict of interest

The authors declare that there is no conflict of interests.

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Ethical Approval

The protocols used in the study were approved by the Ethics Committee of the Faculty of Medicine, Ain Shams University. The patient's consents and statement of protection of the patient's privacy are provided.

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