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Original Article

Resistance and heteroresistance to colistin among multidrug-resistant and extensively drug-resistant Gram-negative organisms isolated from patients admitted to Zagazig University Hospitals

Salah Al-Sayed Ibrahim Ali[1], Aya A Ghamry[2], Seham K Khirala[2], Asmaa M El-Nasser[2] Fatma M Attia Al-Sayed[3], Fatma Atef Ibrahim[1]

[1] Clinical Pathology Department, Faculty of Medicine, Zagazig University, Sharqia, Egypt
[2] Medical Microbiology and Immunology Department, Faculty of Medicine (for Girls), Al-Azhar University, Cairo, Egypt
[3] Internal Medicine Department. Faculty of Medicine, Zagazig University, Sharqia, Egypt

Abstract

Introduction: Nowadays, treating serious infections caused by multi-drug resistant Gram-negative bacteria is best left to the antiquated medication "colistin.". There have been reports of colistin-resistant (Col-R) and heteroresistant (hR) MDR and XDR-GNB strains worldwide. Therefore, we aimed to ascertain the rate of colistin resistance, certain potential resistance mechanisms, and heteroresistance in colistin-susceptible (Col-S) clinical isolates.

Methodology: Identification and Antibiotic susceptibility test (AST) for all isolates were determined by Vitek-2 automated system. The Col-S strains were evaluated for heteroresistance using the population analysis profiling (PAP) method, while the Col-R strains were tested for mcr-1 gene activity by combined disk test (CDT) and colistin minimum inhibitory concentration reduction (CMR) test. The efflux pump mechanism was identified using cyanide 3-chlorophenylhydrazone (CCCP).

Results: Out of 60 isolates enrolled in the study, AST revealed that 60% were MDR-GNB and 40% were XDR-GNB. Ten isolates were colistin resistant (16.6%). The mcr-1 gene was detected in five (5/10) Col-R isolates by PCR. CDT test detected mcr-1 gene activity in four (4/5) of mcr-1 gene positive isolates, while CMR test detected all. Efflux pump inhibition by CCCP showed a reduction of MICs by ≥ 8-folds in four Coli-R isolates. The frequency of carbapenem resistance (CR) within Col-hR strains was 75%, while ESBL was 25%.

Conclusions: The alarmingly high occurrence of colistin resistance and heteroresistance in hospital care settings is of major concern and necessitates a reassessment of recommended AST methods since it can result in colistin therapy failure.

Key words: Colistin; CRE; MDR; XDR; GNB.

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Introduction

The World Health Organization (WHO) has reported that multidrug-resistant Gram-negative bacteria (MDR-GNB) infections have become a major global concern for healthcare providers in recent times due to their significant increase. Because of this, infections may not respond to treatment, raising medical expenses and lengthening hospital stays [1,2].

There are few clinically available treatment options for MDR-GNB infections, particularly those brought on by Enterobacteriaceae that produce carbapenemases and extended-spectrum beta-lactamases (ESBL) [3]. In these situations, one of the most often prescribed antibiotics of choice for treatment is an old drug "colistin" [4].

Although colistin is a bactericidal antibiotic, its use was temporarily restricted due to its nephrotoxicity and neurotoxicity, it has recently gained significant clinical importance on a global scale, and it is considered a last resort for treating severe infections by GNB, particularly those caused by MDR and XDR strains of *Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia*, and *Enterobacteriaceae* [5].

While not all of the mechanisms underlying colistin resistance (Col-R) have been identified, lipopolysaccharide (LPS) modifications in GNB have been linked to the development of colistin resistance by obstructing fixation, losing target, hiding binding sites, or trapping polymyxins through excessive production of capsule polysaccharide. Additionally, it has been reported that certain alterations to outer membrane porins and overexpression of efflux pump systems [6].

Multiple chromosomal mutations are primarily responsible for acquired resistance to colistin [6]. Regretfully, the overuse of colistin in both human and veterinary medicine has led to the emergence of plasmid-mediated *mcr*-genes 1-10 [7,8].

While the broth microdilution method (BMD) was jointly recommended by The Clinical & Laboratory Standards Institute (CLSI) and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) as the reference method for minimal inhibitory concentration (MIC) determination of colistin [9]. Colistin heteroresistance (Col-hR) cannot be detected by manual BMD [6]. Heteroresistance is the existence of resistant subpopulations in an isolate that is susceptible to colistin by in vitro susceptibility tests [10]. The failure of colistin therapy may be due to the presence of Col-hR in GNB [5]. Population Analysis Profiling (PAP) is the current standard method for detecting Col-hR; however, it is a time-consuming and laborious process [11].

The high prevalence of infectious diseases and the lack of restrictions on the use of antibiotics in veterinary care and medicine make the emergence of colistin resistance in Egypt extremely concerning [12]. Therefore, the study aimed to investigate MDR-GNB and XDR-GNB isolates, in order to ascertain the rate of colistin resistance as well as some potential resistance mechanisms and heteroresistance in colistin-susceptible (Col-S) clinical isolates.

Methodology

Study design

This retrospective cross-sectional study was conducted in the Microbiology Unit of Clinical Pathology Department, Zagazig University hospitals (ZUHs), Egypt, over 4 months, from November 2023 to February 2024. The study protocol was approved by Zagazig medical research ethical committee (IRB: 11243-8-11-2023). Informed consent was taken from each patient to use their data in the current research.

Subjects

Of a total of 650 non-repetitive samples obtained from patients admitted at ICUs of internal medical and surgical wards of ZUHs, as part of standard hospital-laboratory procedures, 60 isolates were identified as either MDR (n = 36) or XDR (n = 24), and none as PDR-GNB. The samples enrolled in our study were from respiratory tract infections, surgical site infections, bloodstream infections and ear discharge. The patients of the study were 55% females and all age groups were involved.

Bacterial isolation and identification

All clinical samples were cultured on blood agar and MacConkey agar plates (OXOID) and incubated overnight at 35–37 °C, for blood cultures, samples were inoculated and incubated in BacT/ALERT 3D automated system. Identification of isolates was determined with Vitek-2 automated platform (bioMérieux, Marcy l'Etoile, France) using the GN-ID card, according to the manufacture instructions and following CLSI guidelines [13]. The 60 isolates of this study included *K. pneumoniae* (n = 27), *Pseudomonas aeruginosa* (n = 16), *Escherichia coli* (n = 12) and *Acinetobacter baumannii* (n = 5).

Antibiotic susceptibility test (AST)

The AST against different classes of antibiotics including colistin was performed by Vitek-2 using the AST-XN05 card. MIC data for each organism were interpreted according to the CLSI [13] and the VITEK 2 Advanced Expert System (AES). Colistin susceptibility was assessed using breakpoints suggested by EUCAST [14]. The European Centre for Disease Prevention and Control (ECDC) and the CDC proposed standardized international terminology, which served as the basis for the definitions of MDR, XDR, and PDR strains [15]. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used for quality control. Following AST, every isolate was kept at -20 °C in tryptic soy broth (TSB) with 20% glycerol until they were processed further.

Detection of colistin resistance

A. Combined Disc Diffusion Test (CDT)

It is a phenotypic test that ascertains the mcr-1 phosphoethanolamine transferase activity of the colistin resistance gene (mcr-1 gene). All colistin-resistant isolates were tested using 100 mM EDTA (Sigma-Aldrich; St.268 Louis, MO, USA) to inhibit the mcr-1 activity because this concentration showed no antimicrobial activity. The bacterial strains were cultivated on Muller-Hinton agar (Oxoid, UK) with the addition of three discs. One disc was treated with 10 µL of 100 mM EDTA to make sure the concentration of EDTA used did not prevent the growth of bacteria, the other two remaining discs were treated with 10 µg of colistin and 10 µg of colistin mixed with 10 µL of 100 mM EDTA. When compared to the colistin disc, the isolates showed an increase in the inhibition zone diameter of the colistin/EDTA disc of about 3 mm [16].

B. Colistin MIC Reduction (CMR) assay

The mcr-1 gene, which codes for colistin resistance, is tested phenotypically at the MIC level. A decrease in colistin MIC by ≥ 4-fold among MCR-1-positive isolates was observed in the presence of EDTA, which was made possible by the BMD method's fixing of the final concentration of EDTA at 80 µg/mL because no colistin-resistant screened isolates demonstrated any antibacterial activity at this concentration [16].

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

The *mrc-1* gene was tested in all ten isolates that were resistant to colistin. A single colony was dissolved in 200 µL of distilled water, which was then lysed at 100 °C for five minutes to prepare the DNA template. The supernatant was then collected by centrifugation, and it was diluted 1:10 in Tris HCl buffer for use [17]. Polymerase chain reaction (PCR) was carried out using previously published primers, and the result was examined in accordance with the laboratory protocol optimized by the National Food Institute, Denmark, for the detection of plasmid-mediated Col-R genes [17]. Gel 2% electrophoresis was carried out for thirty minutes. UV light was used to visualize and compare PCR amplicons with a DNA ladder.

D. Efflux Pumps Inhibition by CCCP

In accordance with CLSI guidelines [13] the cation-adjusted Mueller-Hinton broth (Sigma-Aldrich, St Louis, USA) was used for the broth microdilution method to determine the MICs. The MICs of isolates resistant to colistin, both with and without cyanide 3-chlorophenylhydrazone (CCCP), were ascertained. CCCP was prepared as a 5 mg/mL stock solution by dissolving it in 50% methanol (v/v) and keeping it shielded from light. A series of 2-fold-dilutions of CCCP and colistin were detected for the resistant isolates by Vitek 2. The effect of CCCP on colistin MIC was measured using sub-MIC; the antibiotic's MIC concentrations were serially increased while CCCP's (0.5 × MIC) concentration was maintained at the same levels. A sub-MIC of CCCP (final concentration of 10 mg/L) was used to calculate the MICs of the isolates to colistin in the presence and absence of the compound. After being thawed, the strains were inoculated onto agar blood plates and incubated at 35 °C for 18 to 24 hours. The BMD plates were then inoculated with a suspension of a 0.5 McFarland standard. Before the values were read, the plates were incubated for 18 to 24 hours at 35 °C at room temperature [18]. The ratio of the MIC level of the CCCP-free antibiotic to that of the CCCP-added antibiotic was used to compute the resulting MIC fold changes following the addition of CCCP. A ≥ 8-fold decrease in colistin MIC following the addition of CCCP was the positive criterion, for the presence of efflux pumps in isolates [19].

Determination of heteroresistance to colistin by PAP test

A PAP protocol adapted from Liao *et al.* [20] was used to evaluate all isolates with MIC values for colistin ≤ 2 ug/mL for Col-hR. In short, 50 µL of the serial 10-fold saline dilutions ranging from 10^8 to 10^2 CFU/mL of the bacterial suspension were added to cation-adjusted Mueller-Hinton agar plates that contained 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, and 16 µg/mL of colistin sulphate (Thermofisher Scientific). The bacterial suspension was prepared from overnight culture for each isolate and adjusted to an inoculum of roughly 10^8 CFU/mL in saline (0.5 McFarland). Following a 48-hour incubation period at 37 °C, colonies were tallied. Col-hR isolates were identified as those that contained subpopulations that could proliferate when exposed to more than 2 µg/mL of colistin [21]. The frequency of hR-subpopulations was calculated by dividing the number of colonies on the plate with the highest colistin concentration by the number of colonies on a colistin-

Table 1. Demographic characteristics of studied patients infected with Multidrug retistant and Extensively drug resistant-Gram negative bacteria (N = 60).

Characteristics	N	%
Age (y)		
Range	1 – 86	
Mean ± SD	41.6 ± 19.11	
Infants < 2 yrs.	2	3.30%
Children & adolescent	8	13.30%
Adult	18	30.00%
Old age	32	53.30%
Gender		
Male	27	45.00%
Female	33	55.00%
Organisms		
Klebsiella pneumoniae	27	45.00%
Pseudomonas aeruginosa	16	26.70%
Escherichia coli	12	20.00%
Acinetobacter baumannii	5	8.30%
Departments		
ICUs	30	50.00%
medical departments	25	41.70%
surgical department	5	8.30%
Clinical samples		
Respiratory tract infections	36	60.00%
surgical site infections	17	28.40%
bloodstream infections	5	8.30%
ear discharge	2	3.30%
Resistance pattern		
MDR-GNB	36	60.00%
XDR-GNB	24	40.00%
Resistance pattern		
CR	45	75.00%
ESBL	13	21.70%

Table 2. Distribution of studied resistant mechanisms for colistin resistant isolates.

Isolates	MCR-1 (Gene/ activity)			MICs of colistin resistant strains by BMD (µg/mL)	EPI by CCCP
	MCR-1	CDT	CMR		MICs for colistin with CCCP by BMD (µg/mL)
K. pneumoniae	-	-	16 (no change)	16	2 (8 fold)
K. pneumoniae	+	+	1 (8 fold)	8	8 (no change)
P. aeruginosa	-	-	16 (2 fold)	32	4 (8 fold)
P. aeruginosa	-	-	8 (2 fold)	16	8 (2 fold)
P. aeruginosa	+	+	2 (8 fold)	16	16 (no change)
P. aeruginosa	+	-	2 (4 fold)	8	4 (2 fold)
Escherichia coli	+	+	4 (16 fold)	64	4 (16 change)
Escherichia coli	+	+	4 (8 fold)	32	32 (no change)
A. baumannii	-	-	32 (no change)	32	16 (2 fold)
A. baumannii	-	-	32 (2 fold)	64	4 (16 fold)

A. baumannii: Acinetobacter baumannii; P. aeruginosa: Pseudomonas aeruginosa; CDT: Combined Disc Diffusion Test; CMR: Reduction of colistin MIC; EPI: Eflux Pumps Inhibition Broth microdilution; CCCP: cyanide 3-chlorophenylhydrazone.

free plate [22]. To test the stability of the heteroresistant phenotype, a single colony from each isolate that had the highest antibiotic concentration was chosen, and the colistin MIC was reevaluated after five days of serial passaging on an antibiotic-free medium [23].

Results

Out of 650 non-repetitive samples isolated from different clinical specimens of hospitalized patients in ZUHs during the study period, 60 GNB (MDR or XDR-GNB) isolates detected by Vitek-2 were enrolled in the current study. The age of studied patients ranged from 1 to 86 years. Majority of the patients were females (55%). The bulk of clinical samples were from respiratory tract infections, while the majority of isolates were obtained from intensive care units. *Klebsiella pneumoniae* was the most common isolate, and the majority of the isolates were resistant to carbapenem (meropenem) (Table 1). Table 2 revealed some colistin resistance mechanisms: Mcr-1 gene was detected by PCR in five isolates, while CDT test phenotypically detected four (4/5) of mcr-1 gene positive isolates. The enzymatic activity of Mcr-1 gene by CMR assay was detected in all col-R isolates with the Mcr-1 gene. Efflux pump inhibitor (EPI) was determined using CCCP assay by MIC Reduction using 0.5 MIC of CCCP to study its effect on colistin MICs, 4/10 isolates were found to have efflux pump mechanism. One Escherichia coli isolate was found to be positive for mcr-1 gene by PCR, CDT, CMR, and efflux mechanism (Table 2). In Table 3, out of the 60 Gram-negative isolates, 60% were MDR (36/60), and 40% were XDR (24/60). 27 isolates were *K. pneumoniae* (22 MDR and 5 XDR), 16 isolates were *P. aeruginosa* (6 MDR and 10 XDR), 12 isolates were *E. coli* (3 MDR and 9 XDR), and all *A. baumannii* isolates were MDR. Also, 16.6% (10/60) were colistin resistant (2 isolates from each of *K. pneumonia, E. coli* and *A. baumannii* isolates and 4 col-R isolates were *P. aeruginosa*. Moreover, 24% (12/50) of Col-S isolates were colistin heteroresistant (6 isolates were *K. pneumonia*, 4 isolates were *P. aeruginosa*, 1 isolate was *E. coli* and 1 isolate was *A. baumannii*. Table 4 showed that out of 50 Col-S isolates, 12 (24%) isolates were Col-hR, among the heteroresistant strains 75% (9/12) were CR and 25% were ESBL producers. *K. pneumoniae* displayed the great proportion among Col-hR strains 50% (6/12). The frequency of Col-hR subpopulations ranged from 4×10^{-5} to 6.2×10^{-4}. The MICs for the Col-hR strains by Vitek 2 ranged from > 0.5 –2 ug/mL, while the maximum concentration at which heteroresistance strains can grow in PAP method ranged from 4-16 ug/mL. After five days of passages of the resistant subpopulations on antibiotic-free medium (MHA) plates, only one hR strain (8.3%) showed stable resistance to colistin with MIC by BMD of ≥ 64 µg/mL.

Discussion

According to a World Health Organization (WHO) fact sheet, MDR-GNB infections have become a major global concern for healthcare providers in recent years, with a marked increase in cases [2].

In our study, out of 650 non-repetitive samples, 60 GNB isolates were either MDR (n = 36/60; 60%) or

Table 3. Summary of frequency and distribution of the resistance pattern among resistant Gram-negative isolates.

Isolates	MDR-GNB, 36/60		XDR-GNB, 24/60		Col-R, 10/60		Col- hR, 12/50	
	No	%	No	%	No	%	No	%
Klebsiella pneumoniae (27)	22	36.7	5	8.3	2	3.3	6	12
Pseudomonas aeruginosa (16)	6	10	10	16.7	4	6.7	4	8
Escherichia coli (12)	3	5	9	15	2	3.3	1	2
Acinetobacter baumannii (5)	5	8.3	-	-	2	3.3	1	2
Total	36	60	24	40	10	16.6	12	24

XDR (n = 24/60; 40%). These results agree with other Egyptian studies reported that MDR was 61.3%, 55.1% and XDR was 38.7%, and 34.7% respectively [24,25]. A different study conducted in South India stated that 66% out of 150 isolates were MDRO [26].

The result of a study conducted in Saudi Arabia by Alkofide *et al.* [27], were in accordance with our findings with reference to MDR-GNB at 57.3% and weren't per our results for XDR, which was 3.5%. However, in Oman, a study reported that only 36% of the strains were MDR-GNB (63/175 isolates) [28]. In a study conducted in Nepal, higher rates of MDRO were found; nearly 96% of the GNB isolates were found to be MDRO, and 43.3% of isolates were XDR [29]. Also in Egypt, a study by Abd El-Baky *et al.*, reported that MDR and XDR isolates were 96% and 87%, respectively [30].

As colistin is so widely used, more resistant strains are being found in clinical settings [31]. In our study, colistin resistance (Col-R) was 16.6%. This result matched previous studies that reported col-R 19.9% and 15%, respectively [32,33]. The presence of colistin resistance in MDRO identified in our investigation may be the consequence of inadequate infection control protocols and frequent colistin use as a result of limited treatment alternatives. Colistin was deemed a critically important antibiotic in human medicine in 2019 by the European Medicines Agency (EMA) under Category B- "Restrict." This means that in order to lessen the risk to the public's health, administration in animals should be restricted [34].

It has been reported that mechanisms of colistin resistance are only chromosomally mediated. Nonetheless, the discovery of the mcr-1 transferable plasmid-mediated colistin resistance gene raises the prospect of quick resistance acquisition [35], reduction of the negative charges found in LPS by addition of phosphoethanolamine to the phosphate group of the lipid A moiety anchored on LPS gave the LPS resistance to polymyxins [36].

In our study the mcr-1 gene was detected in five Col-R isolates including one *K. pneumoniae* isolate and two in each of *P. aeruginosa* and *Escherichia coli* isolates. This result was consistent with an earlier study reported that the mcr-1-containing plasmid was found in many parts of the world among Enterobacterales and non-fermentative GNBs [37]. Moreover, other studies done in Egypt and in Iran displayed positive results for mcr-1 gene [30,38]. However, others reported that none of the colistin resistant *Enterobacteriaceae* isolates were positive for mcr-1 gene and all non-fermentative GNBs were colistin susceptible [39].

In our study, combined disc diffusion test (CDT) and the Reduction of colistin MIC assay (CMR) by EDTA were used as phenotypic methods for the detection of mcr-1 gene activity. Out of 5 mcr-1 positive isolates, 4 isolates were positive for CDT while one mcr-1 positive (*P. aeruginosa* strain) was negative for CDT which may be due to the low and variable diffusibility of colistin from disks into the Mueller-Hinton agar, as previously reported by Coppi *et al.* [40]. Using the CMR assay, all isolates that tested positive for mcr-1 and resistant to colistin showed a ≥ 4-fold reduction in colistin minimum inhibitory concentration when EDTA was present. Nevertheless, some other reports stated that in the presence of EDTA, some mcr-1-negative (colistin-susceptible and colistin-resistant) strains showed a ≥ 4-fold colistin MIC decrease, and some mcr-1-positive (colistin-susceptible and colistin-resistant) isolates only showed a 2-fold colistin MIC decrease [16].

Additionally, we used CCCP, an efflux pump inhibitor, to look for the presence of an efflux pump mechanism in isolates resistant to colistin. According to our findings, four isolates had an efflux mechanism, and one of them tested positive for mcr-1 through PCR,

Table 4. Population analysis profiling (PAP) of CRE isolates with colistin heteroresistance.

Species	Associated resistances	MIC by Vitek 2 µg/mL	PAP µg/mL	MIC by BMD of hR strains after Passages (µg/mL)	Frequency	Strain type
Klebsiella pneumoniae	CR	> 0.5	4	> 0.5	3.8×10^{-4}	hR
Klebsiella pneumoniae	CR	1	10	1	5.1×10^{-4}	hR
Klebsiella pneumoniae	CR	1	6	1	2.0×10^{-4}	hR
Klebsiella pneumoniae	CR	0.5	10	0.5	4.5×10^{-4}	hR
Klebsiella pneumoniae	ESBL	2.0	8	2.0	1.0×10^{-4}	hR
Klebsiella pneumoniae	ESBL	> 0.5	12	> 0.5	2.5×10^{-4}	hR
Pseudomonas aeruginosa	CR	1	10	1	6.2×10^{-4}	hR
Pseudomonas aeruginosa	CR	2	16	> 64	7.0×10^{-5}	hR
Pseudomonas aeruginosa	CR	1	8	1	5.0×10^{-4}	hR
Pseudomonas aeruginosa	CR	2	4	2	2.6×10^{-4}	hR
Acinetobacter baumannii	ESBL	0.5	8	0.5	4.0×10^{-5}	hR
Escherichia coli	CR	1	16	1	2.8×10^{-4}	hR

CR: carbapenems resistance; ESBL: Extended spectrum β-lactamase; hR: heteroresistance.

CDT, and CMR. These findings aligned with a different investigation that demonstrated an efflux mechanism in three isolates, one of which was mcr-1 positive [30]. Furthermore, it was demonstrated by others that over 50% of isolates resistant to colistin had efflux pumps [41]. These findings could help to explain why a single isolate has multiple colistin resistance mechanisms.

Heteroresistance has been known as the presence of resistant subpopulations by in vitro susceptibility tests in an isolate that is susceptible to colistin [10]. MIC for colistin is 2 ug/mL as per the guidelines set forth by EUCAST, and the colistin optimal plasma concentration for treating such infections is around 2 ug/mL, the rate at which these subpopulations can grow and cause treatment failure [5].

In the current study, colistin-hR was detected by PAP test in 24% (12/50) of Col-S isolates with the highest proportion detected among *K. pneumonia* (50%, 6/12), followed by *P. aeruginosa* (33.3%, 4/12), *E.coli* and *A. baumannii* (8.3%, one for each). In other reports, hR was detected in 10.1% (41/408) of isolates, where *Enterobacter* spp. displayed the highest proportion [42]. While others detected heteroresistance with variable results of 71.9% in CRKP strains and 1.37% in *E.coli* strains respectively [20,43]. These results report that the prevalence of colistin- hR varies according to isolation sources, test methods, standards, and previous colistin exposure [44]. Moreover, in this study carbapenem resistance was displayed in 75% (9/12) of colistin- hR strains. Only one strain of resistant subpopulations 8.3% (1/12) showed stable resistance to colistin after five days passages onto colistin-free MHA plates, with MIC ≥ 64 ug/mL. Another report just detected 7.3% of Col-hR strains classified as colistin-resistant by BMD [42]. Proving that in the absence of antibiotic pressure, an unstable subpopulation can return to the original population's susceptibility pattern [10]. On the other hand, another study reported that all *Coli*-hR showed stable resistance to colistin explained by overexpression of pmrD and phoPQ [43].

Conclusions

The emergence of colistin resistance is of great concern in hospital setting. The alarmingly high rate of colistin heteroresistance isolates necessitates a reassessment of advised AST techniques since it can result in colistin treatment failure.

Corresponding author

Aya Ahmed Ghamry
Medical Microbiology and Immunology Department
Faculty of Medicine (for Girls), Al-Azhar University
Cairo, Egypt.
Tel: 01093355643
E-mail: ayaahmed.micro@azhar.edu.eg

Conflict of interests

No conflict of interests is declared.

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