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ORIGINAL ARTICLE

Study of *mcr-1* Gene-Mediated Colistin-Resistance in Gram-Negative Isolates in Egypt

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ABSTRACT

Background: *The re-use of colistin (last-resort drug) to treat infections caused by resistant Gram-negative bacteria, has led to the emergence of a serious resistance against colistin. A new transferable plasmid-mediated colistin-resistance gene (mcr-1) has been described globally. Screening for such gene will provide an aiding step to explore the extent of colistin-resistance in Egypt.* ***Objectives:*** *To isolate the causative Gram-negative bacteria from different hospital-acquired (HA) and community-acquired (CA) infections, determine the antibiotic susceptibility pattern of the isolated bacteria, detect colistin-resistance and investigate the existence of mcr-1 gene in colistin-resistant isolates.* ***Methodology:*** *This study was carried out on 400 patients with HA- and CA-infections. Samples were taken from sputum, endotracheal aspirates, bronchoalveolar lavage, surgical and burn wounds, stool and blood. Bacterial isolation and identification were done by standard microbiological methods. Colistin-resistance was assessed by broth macrodilution method, then mcr-1 gene was detected in colistin-resistant isolates by conventional polymerase chain reaction (PCR).* ***Results:*** *Gram-negative organisms were the commonest isolates in both HA (67.6%) and CA (79.4%) infections. Colistin-resistance was detected in only 10 cases. mcr-1 gene was not detected in any of the tested colistin-resistant isolates.* ***Conclusion:*** *The prevalence of colistin-resistance in the study cases is still low and has not extended to the community yet. Colistin intake is not a prerequisite for the occurrence of resistance, but could be a supporting factor.*

INTRODUCTION

Antimicrobial resistance represents one of the most critical global threats to human health in this century. WHO and US Centers for Disease Control and Prevention expect a worldwide disaster and an imminent danger of a return to the pre-antibiotic era.[1] Multidrug resistant (MDR) bacteria have emerged in both hospitals and community, meaning that reservoirs of antibiotic resistant bacteria are existing also outside the hospitals.[2]

Polymyxins, recently reintroduced in human medicine, represent one of the last options for the treatment of MDR Gram-negative bacteria. Colistin (polymyxin E) is a polycationic peptide that bind to anionic lipopolysaccharide molecules of the outer membrane of the Gram-negative cell wall causing its disruption, through a competition with Ca^{2+} and Mg^{2+} cations.[3,4] However, there may be other unclear mechanisms of colistin action. [5]

The re-use of colistin has led to the emergence of colistin-resistance. Acquired colistin-resistance is commonly due to chromosomal mutations, however, a new transferable plasmid-mediated colistin-resistance gene (*mcr-1*) encoding phosphoethanolamine transferase enzyme, has lately been discovered in late 2015.[1,6] This enzyme modifies the lipid A of the outer membrane lipopolysaccharides.[7] Resistance transmitted by plasmids has two hazards. First, plasmids can convey resistance to multiple antibiotics. Second, plasmids can spread resistance into the bacteria at a greater rate than occurs via spontaneous mutation. So, colistin-resistant bacteria may rapidly become endemic in the world in absence of new antibiotics against resistant Gram-negative bacteria.[8, 9]

mcr-1 gene was first discovered in an *Escherichia coli* in China, then it was detected nearly worldwide in ≈10% of animal isolates[10] and in 0.1%–2% of human isolates.[11] This indicates that this plasmid-mediated resistance spread well from animals (where colistin was used for long time as a treatment or growth promotor) to humans through horizontal gene transfer. Also, the *mcr-1* gene was found in different Gram-negative bacteria, including *Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, Enterobacter, Salmonella* and *Citrobacter*.[12]

In addition, *mcr-1* gene has been discovered in many countries across five continents. This gene has been spread within hospital environments although not used

and also in the community.[13] So, the aim of this study was to isolate the causative Gram-negative bacteria from different hospital-acquired (HA) and community-acquired (CA) infections, determine the antibiotic susceptibility pattern of the isolated bacteria and investigate the existence of *mcr-1* gene in colistin-resistant isolates.

METHODOLOGY

Subjects:

This study was carried out in Medical Microbiology and Immunology Department, Faculty of Medicine, Tanta University, Egypt on 400 patients admitted during the period of the research (March 2017 to February 2018) to Medical and Surgical Intensive Care Units, Burn unit, Nephrology unit and Outpatient clinics of Tanta University Hospitals.

The study design was approved by the Ethics Committee, Faculty of Medicine, Tanta University.

Sampling:

The samples were taken from sputum, endotracheal aspirates, bronchoalveolar lavage, surgical and burn wounds, stool and blood under complete aseptic precautions.

Inclusion Criteria

- Age: more than 18 years.

Exclusion Criteria

- Patients showed good response to antibiotic therapy.

Isolation and identification of the infecting organisms

Blood, XLD, CLED and MacConkey's agar plates were used according to the type of sample. All plates were incubated aerobically at 37°C for 24-48 h. The bacterial growth was identified by the routine microbiological methods.

Antibiotic sensitivity testing

Antimicrobial susceptibility of the isolates was determined by modified Kirby-Bauer disc diffusion method on Mueller-Hinton agar plates according to the Clinical and Laboratory Standard Institute (CLSI) guidelines.[14] Gram-negative organisms were tested against the followings: imipenem (10 µg), meropenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ceftazidime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), cefepime (30 µg), cefotaxime (30 µg), aztreonam (30 µg), amoxicillin / clavulanic acid (20/10 µg), sulfamethoxazole / trimethoprim (1.25/23.75 µg), ampicillin (10 µg), pipracillin / tazobactam (100/10 µg), ciprofloxacin (5 µg) and levofloxacin (5 µg). Nalidixic acid (30 µg), norfloxacin (10 µg) and nitrofurantoin (300 µg) were used only in urine samples.

Phenotypic detection of colistin-resistance:

Minimal inhibitory concentration (MIC) of colistin was determined by broth macrodilution method.[15] According to European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations, isolates with MIC > 2 mg/L were considered resistant and MIC ≤ 2 mg/L were considered sensitive. [16]

Genotypic detection of colistin-resistance mcr-1 gene:

DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega). *mcr-1* gene was detected by conventional polymerase chain reaction (PCR) as described by Cavaco L& Hendriksen R. [17] GoTaq®Long PCR Master Mix amplification kits (Promega) were used. The used primers were CLR F (5'CGGTCAGTCCGTTTGTTC'3) and CLR R (5'CTTGGTCGGTCTGTAGGG'3).[1]

Colistin-resistant *mcr-1* positive *E.coli* (NCTC 13846) was used as a control strain.

Statistical analysis

Quantitative variables were described in the form of mean ± SD and were analyzed using Student's t test, while categorical data were presented as numbers and percentages and were analyzed using chi-square ($\chi 2$) test. Fisher's exact test was used to test differences between the nominal data (frequencies). *P* values < 0.05 were considered significant**.**

RESULTS

Distribution of cases:

Overall, 400 patients were recruited in this study, 300 patients were suffering from HA-infections (group 1), while the other 100 patients had CA-infections (group 2). Demographic characteristics of both study groups are shown in table 1. Among the included subjects, there was 232 males and 168 females with a mean age of 48.2±10.7 years. There were no significant differences between the 2 study groups according to age or gender. The most frequent type of samples withdrawn from patients in both study groups were sputum samples representing (31.7%) in group 1 and (35%) in group 2, followed by urine representing (23.3%) in group 1 and 30% in group 2 then septic wound swabs representing (10%) in group 1 and (20%) in group 2. Endotracheal aspirates, bronchoalveolar lavage and blood samples were limited to HA-infections. There was a statistically significant difference between the 2 studied groups regarding the type of sample.

Type of growth:

In HA-infections, (73.7%) of samples showed monomicrobial growth, (10%) showed polymicrobial growth and (16.3%) showed no growth. While, in CA-infection, (58%) of samples showed monomicrobial growth, (5%) showed polymicrobial growth and (37%) showed no growth. There was a statistically significant difference between the 2 studied groups according to the type of growth (Table 1).

Table 1: **Demographic characteristics of both study groups:**

Characteristics	Total (n=400)	HA cases (n=300)	CA cases (n=100)	*P*- value
Age (in years)				
Range	18-80	24-80	18-65	
Mean±S.D	48.2±10.7	48.3±10.5	47.9±11.3	0.751
Gender				
Male	221(55.3%)	172(57.3%)	49(49%)	0.147
Female	179(44.8%)	128(42.7%)	51(51%)	
Samples				
Sputum	130(32.5%)	95(31.7%)	35(35%)	
Endotracheal aspirates	50(12.5%)	50(16.7%)	0(0%)	
Bronchoalveolar lavage	15(3.8%)	15(5%)	0(0%)	
Septic wound swabs	50(12.5%)	30(10%)	20(20%)	
Infected burn swabs	30(7.5%)	20(6.7%)	10(10%)	
Urine	100(25%)	70(23.3%)	30(30%)	
Stool	15(3.8%)	10(3.3%)	5(5%)	<0.001*
Blood	10(2.5%)	10(3.3%)	0(0%)	
Types of growth				
Mono-microbial	279(69.8%)	221(73.7%)	58(58%)	
Poly-microbial	35(8.8%)	30(10%)	5(5%)	<0.001*
No growth	86(21.5%)	49(16.3%)	37(37%)	

* P<0.05 was considered significant. HA, hospital-acquired; CA, community-acquired

Bacterial outcome:

Gram-negative organisms were the commonest isolates in both study groups (69.9%), representing 67.6% in group 1 and 79.4% in group 2. Gram-positive organisms (25.2%) were detected in 26.3% in group 1 and 20.6% in group 2. Fungi were detected only in group 1 (6%). There was a statistically significant difference between the 2 studied groups regarding bacterial outcome. *K. pneumoniae* was the most frequent Gram-negative isolates in both study groups representing 43.4%, followed by *E. coli* (29.1%), then *P. aeruginosa* (13.5%). Both groups are statistically homogenous concerning Gram-negative isolates table 2.

Table 2: Bacterial outcome in both study groups:

Organisms	Total (n=349)	HA isolates (n=281)	CA isolates (n=68)	*P*- value
Fungi	17(4.9%)	17(6%)	0(0%)	0.04*
Gram-positive	88(25.2%)	74(26.3%)	14(20.6%)	
Gram-negative	244(69.9%)	190(67.6%)	54(79.4%)	
Klebsiella pneumoniae	106(43.4%)	83(43.7%)	23(42.6%)	0.913
Escherichia coli	71(29.1%)	52(27.4%)	19(35.2%)	
Pseudomonas aeruginosa	33(13.5%)	26(13.7%)	7(13%)	
Acinetobacter baumannii	13(5.3%)	12(6.3%)	1(1.9%)	
Enterobacter spp	5(2%)	4(2.1%)	1(1.9%)	
Citrobacter	2(0.8%)	2(1.1%)	0	
Proteus	12(4.9%)	9(4.7%)	3(5.6%)	
Serratia	1(0.4%)	1(0.5%)	0	
Morganella morgagni	1(0.4%)	1(0.5%)	0	

* P<0.05 was considered significant. HA, hospital-acquired; CA, community-acquired

Drug resistance pattern in Gram-negative isolates:

The results revealed that of all 244 culture-confirmed Gram-negative isolates, 150 (61.5%) were considered MDR (defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories) [18], while 29.5% were XDR (defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories).[18]

Phenotypic detection of colistin-resistance:

Proteus, *Serratia* and *Morganella* were intrinsically resistant to colistin, so they were excluded. MIC of colistin for 61 Gram-negative isolates was detected by broth macrodilution method. Only 10 (16.4%) isolates were resistant to colistin. All resistant isolates were from HA-infections. Among the 10 resistant isolates, 8 were *K. pneumoniae* and only one *E. coli* isolate and one *P. aeruginosa*.

Clinicomicrobiological profile of the 10 patients with colistin-resistant isolates:

Respiratory samples (50%) were the most prevalent, followed by urine (30%) and wound swabs (20%). History of colistin intake was positive in 2 cases (20%). Antibiotics that were used in their current admission before isolating colistin-resistant organism were imipenem, cefotaxime, ceftriaxone, cefepime and ciprofloxacin. The most common isolated organisms were *K. pneumoniae* (80%), followed by *E. coli* (10%) and *P. aeruginosa* (10%). Majority of cases were sensitive to aminoglycosides (80%), followed by carbapenems (70%). Sensitivity to ciprofloxacin, piperacillin/tazobactam and nitrofurantoin was only (10%) for each one. Death occurred in 4 cases (40%), while 4 cases (40%) were improved and discharged. The remaining 2 cases (20%) were subjected to surgical management (Table 3).

Table 3: Clinicomicrobiological profile of patients with colistin-resistant isolates

No. 10 Cases	Age in years/sex	Patient diagnosis	Antibiotic taken	Clinical sample	Isolated organism	History of colistin intake	Sensitivity to other antibiotics	Outcome
1	80/male	Pyelonephritis	Cefotaxime	Urine	*K. pneumoniae*	No	Amikacin Gentamicin	Nephrectomy
2	66/female	Bed sore	Ceftriaxone	Wound swab	*K. pneumoniae*	No	Imipenem Meropenem	Discharge
3	50/male	Accident	No	Tracheal aspirate	*K. pneumoniae*	No	Imipenem Ciprofloxacin Amikacin	Death
4	57/male	Pneumonia	Imipenem	BAL	*K. pneumoniae*	Yes	Amikacin	Death
5	45/male	Postoperative	Ceftriaxone	Tracheal aspirate	*K. pneumoniae*	No	Imipenem Meropenem Gentamicin amikacin	Discharge
6	62/female	UTI	Ciprofloxacin	Urine	*E.coli*	No	Imipenem Meropenem Amikacin Nitrofurantoin	Discharge
7	27/male	UTI	Cefepime	Urine	*K. pneumoniae*	No	Gentamicin Amikacin	Discharge
8	58/female	Diabetic ulcer	Cefotaxime	Wound swab	*K. pneumoniae*	No	Meropenem Gentamicin	Amputation
9	72/male	Chronic chest disease	Imipenem	Tracheal aspirate	*P. aeruginosa*	Yes	Imipenem Meropenem Piperacillin/ tazobactam	Death
10	38/female	Chronic chest disease	No	Tracheal aspirate	*K. pneumoniae*	No	Meropenem Gentamicin Amikacin	Death

UTI, urinary tract infection; BAL, bronchoalveolar lavage

Genotypic detection of colistin-resistance (*mcr-1*) gene:

mcr-1 gene was not detected in any of the 10 colistin-resistant isolates by conventional PCR.

DISCUSSION

Colistin is considered a critically important antimicrobial in humans due to its efficacy against MDR Gram-negative bacteria.[19] The emergence of these bacteria has influenced practice in all fields of medicine and are also becoming widespread in the community.[20]

In the present study, Gram-negative organisms were the commonest isolates in both study groups (69.9%), representing (67.6%) of HA-infections and (79.4%) of CA-infections. *K. pneumoniae* was the most frequent Gram-negative isolates in both study groups, representing (43.4%), followed by *E. coli* (29.1%), then *P. aeruginosa* (13.5%). In agreement with this result, Fakhr & Fathy[21] found that among 48 isolates from HA-infections, (60.4%) were Gram-negative isolates. Also, ML & Raja[22] found that out of 777 isolates, (73.5%) were Gram-negative isolates. Among them, the most common isolates were *Klebsiella* spp (37.4%), followed by *E. coli* (24.5%) and *Pseudomonas* (13.6%). In addition, Bhuyan *et al.*[23] found that out of 339 Gram-negative isolates from both HA-infections and CA-infections, *Klebsiella spp* (55.5%) was the most common isolates, followed by *E. coli* (23.9%) and *Pseudomonas spp* (16.8%). On the contrary, *E. coli* was the most frequent Gram-negative isolates in other studies; Kaur *et al.* [24] found that out of 276 Gram-negative isolates, *E. coli* (41.6%) was the most frequent organism, followed by *K. pneumoniae* (24%) and *Pseudomonas spp* (17.7%). This divergence in results may be clarified by difference in the type of samples and number of cases, variations in general condition of the patients, or discrepancy between countries. Compliance with infection control measures is another important varying factor.[25]

Regarding phenotypic colistin susceptibility in the current study, MIC of colistin was detected by broth macrodilution method. Broth **micro**dilution (BMD) is the reference susceptibility test method. It is currently the recommended method by the (CLSI) and (EUCAST) for colistin susceptibility testing.[14,16] However, BMD is quite laborious, manual preparation of antibiotic solutions is time consuming and interpretation of the result is quite difficult. It is therefore not adaptable for most clinical microbiology laboratories. Evaluation of colistin broth macrodilution method against BMD showed no false susceptibility result and highest agreement compared to other observed methods.[26,27] All these data support the use of broth macrodilution in the current study instead of BMD.

So, out of 61 Gram-negative isolates tested for MIC, only 10 (16.4%) isolates were resistant to colistin. All resistant isolates were inpatients. No colistin-resistant isolates were detected in outpatients. In agreement with these results, Taneja *et al.*[28] found that out of 50 *A. baumannii* strains, (16%) were resistant to colistin. while, Jayol *et al.*[29] recorded that among 972 *enterobacterial isolates*, (6.2%) were found to be resistant to colistin. On the other hand, Sinirtaş *et al.* [30] *found that among 100 A. baumannii* strains, susceptibility to colistin was (100%).

In the current study, the 10 colistin-resistant isolates were subjected to molecular detection of *mcr-1* gene, but they were found to be negative. Though, the phenotypic resistance of these 10 isolates could be explained by either, the presence of other resistant genes such as *mcr-2* which is another plasmid-mediated gene isolated at Belgium in June 2016[31], or the presence of chromosomal-mediated resistance.[32] In agreement with this result, Tanfous *et al.* [33] showed that colistin *mcr-1* gene was not detected among 24 phenotypically-resistant *K. pneumoniae* isolates. Also, Fernades *et al.* [34] reported that they did not find any *mcr-1* positive isolates among 137 isolates showing phenotypical resistance to colistin.

On the other hand, Wong *et al.*[35] in Hong Kong found that, out of 62 colistin-resistant isolates, 8% of the isolates were *mcr-1* positive. Finding the *mcr-1* gene in Hong Kong may be due to the high amount of livestock and meat imported from China, where prevalence of colistin-resistant isolates is high.[35] Also, Liassine *et al* [9] found that out of 2049 enterobacterial isolates, colistin-resistance was detected in 6 (0.29%) isolates, with only 1 *E. coli* (16.7%) carrying *mcr-1* gene. All their colistin-resistant isolates were HA. They explained this due to the lack of colistin use in treatment of CA-infections. In Egypt, Elnahriry *et al.*[36] found that among 241 Gram-negative isolates collected from different hospitals during 2015, *mcr-1* was detected in only one *E. coli* (0.4%) isolated from sputum of a patient with bacteremia.

Concerning the clinicomicrobiological profile of the 10 patients with colistin-resistant isolates in this study, respiratory samples (50%) were the most prevalent, followed by urine (30%) and wound swabs (20%). This differed from the study of Arjun *et al.* [37], where urine (33%) was the most common source of isolates, followed by blood (25%), respiratory samples (20.8%), pus (16.67%) and cerebrospinal fluid (4.2%). While Qureshi *et al.* [38] isolated their colistin-resistant strains mostly from respiratory samples (85%) which agreed with our results.

In the present study, history of colistin intake was positive in 2 cases (20%). Antibiotics that were used in their current admission before isolating colistin-resistant organisms were imipenem, cefotaxime, ceftriaxone, cefepime and ciprofloxacin. Similarly in Arjun *et al.* [37] study, colistin was previously used by only one out of 24 patients (4.16%). Antibiotics that were used in their relevant admission were colistin, carbapenem, β-

lactam/β-lactamase inhibitor and tigecycline. While a higher percentage was detected in the study of Qureshi *et al*. [38], where (95%) of their cases had received colistin prior to the identification of colistin-resistant isolates. Also, in Goel *et al*. [39] study, (62.5%) of the cases had a present history of colistin intake.

The most common isolated organisms in our ten resistant cases were *K. pneumoniae* (80%), followed by *E. coli* and *P. aeruginosa* (10% for each). This was agreed by Arjun *et al*. [37] and Chen *et al*.[40] where *K. pneumoniae* was the most frequent colistin-resistant isolates at rates of (87.5% and 75%, respectively). On the contrary, in Prim *et al*.[41] study, *K. pneumoniae* was the least frequent colistin-resistant isolates (0.4%), while *Enterobacter* (4.2%) was the most isolated organism.

In the current study, the majority of colistin-resistant cases were sensitive to aminoglycosides (80%), followed by carbapenems (70%), then ciprofloxacin, piperacillin/tazobactam and nitrofurantoin (10% for each). While in Arjun *et al*.[37] study, sensitivity was higher against tigecycline (75%), doxycycline (20.8%) and chloramphenicol (62.5%). Ciprofloxacin was sensitive in only (4.2%) of the isolates.

As regards the outcome of the 10 colistin-resistant cases in this study, death occurred in (40%) of cases, and (20%) of cases deteriorated and required surgical interventions. On the other hand, (40%) of cases were completely improved. Similarly in Arjun *et al*. [37] study, improvement occurred in only (50%) of the resistant cases. While in Goel *et al*. [39] study, (75%) of cases has survived. These variations in the clinicomicrobiological profile between studies could be explained by the difference in the clinical presentations of the patients and their comorbidity, subsequently the variation in the taken samples, types of used antibiotics, and finally the form of provided medical care.

CONCLUSION

The prevalence of colistin-resistance in study cases is still low and has not extended to the community yet. *mcr-1* gene was not detected in any colistin-resistant isolate of the study. Colistin-resistance can occur without history of colistin intake. The outcome of colistin-resistant cases in the study was mostly bad.

Ethical consideration

For consideration as an original article to investigate the existence of *mcr-1* gene in Egypt **.**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 article to be included as author, to the best of my knowledge, no conflict of interest, financial or others exist.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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