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ORIGINAL ARTICLE

Colistin resistance among multidrug-resistant *E. coli* isolated from Upper Egypt

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ABSTRACT

Background: *The increased rate of resistance among Gram-negative bacteria to β-lactam, quinolone, or aminoglycoside antibiotics has resulted in increased use of colistin antibiotic with the unavoidable risk of emerging resistance and spreading of colistin resistant strains.* ***Objectives****: Our study aimed to investigate urinary tract infections for detection of colistin resistance among E. coli strains causing urinary tract infections and isolated from two University hospitals in Upper Egypt; Assiut and Minia University hospitals.* ***Methodology:*** *One hundred isolates of E. coli strains were collected from urine specimens at both Assiut and Minia University Hospitals. Antibiotic sensitivity test was carried out using disc diffusion method. For analysis of colistin resistance, the broth microdilution technique was employed. In addition, the mcr-1 gene encoding an enzyme phosphoethanolamine transferase which has been identified recognized as a source of acquired resistance of colistin was detected by PCR assay.* ***Results:*** *The high resistance pattern among E. coli strains, as most strains were resistant to 13 antibiotics. mcr-1 gene was detected in (20.8%) and (23.1%) of Assiut and Minia University Hospital respectively E. coli isolates. In addition, in this report, we describe the detection of mcr-1 in E. coli isolates causing urinary tract disorders infections in Upper Egypt.* ***Conclusion:*** *Our study revealed a high rate of mcr-1 gene encoding a phosphoethanolamine transferase in E. coli isolates as a source of acquired resistance to colistin. The Emergence of mcr-1 in our isolates of E. coli is alarming. Therefore, broader surveillance of this resistance determinant would be recommended even among other members of Gram-negative bacilli.*

INTRODUCTION

Polymyxins comprise an old group of antibiotics that was discovered in 1947. This group includes different drugs; polymyxins A~E, of which polymyxin E and B (known as colistin) are used clinically. Polymyxins exert their antibacterial action mainly by binding to lipid A fraction of LPS of the organism's cell wall which is followed by insertion of their hydrophobic N-terminal into the outer membrane. This facilities their passage to the inner membrane which is eventually followed by destruction of the strain's phospholipid bilayer of the inner membrane leading to cell lysis and death [1].

Colistin (polymyxin E) is a cationic polypeptide compound which differs only by one amino acid from polymyxin B. Its narrow spectrum of activity is targeted against Gram-negative bacteria. It has a potent antimicrobial activity. Therefore it can be used for the treatment of multidrug-resistant Gram-negative bacterial infections caused by *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* infections. Sometimes it is considered as the last line of treatment of infections by such pathogens[2,3]. It is usually used when other treatment options are invaluable. However, colistin is used only in limited conditions due to their nephrotoxic and neurotoxic side effects [4, 5].

Several strategies have been employed by bacteria to gain resistance to polymyxins. These mechanisms are either intrinsic or chromosomally-mediated including modifications of lipid A with aminoarabinose or phosphoethanolamine, activation of LPS-modifying operon by mutations, inactivation of negative feedback regulators, growing acylation of lipid A with aminoarabinose, trapping of polymyxin by capsule, efflux pump, loss of LPS, glycosylation of lipid A with hexosamine or overexpression of outer membrane proteins[6]. Recently a conjugative plasmid-mediated resistance gene was discovered. The gene is known as *mcr-1* and encodes an enzyme that related to the phosphoethanolamine transferase family. Since its discovery, different studies have reported the *mcr-1* detection that mediated colistin resistance among multidrug-resistant Gram-negative species in different

continents[7-10]. Following its discovery in human isolates, animal strains were screened retrospectively for the existence of *mcr-1* and interestingly, *mcr-1* was detected in *E. coli* isolated from 1980s which was attributed to the employment of colistin in veterinary practice as a therapeutic antibiotic or feed additive in China [11, 12]. The detection of *mcr-1* in old animal samples suggests that *mcr-1* colistin resistance was first developed in animals due to its extensive use in animal production since the mid-1960's which is then transferred to human pathogens [13]. In such study, we describe the level of resistance of colistin in *E. coli* isolates causing UTI and report the first detection of *mcr-1* in colistin-resistant *E. coli* strains from two university hospitals in Upper Egypt.

METHODOLOGY

Bacterial strains:

One hundred clinical isolates of *E. coli* were collected over a period of 12 months between March 2017 and March 2018 from two tertiary hospitals in Upper Egypt. *E. coli* isolates were recovered from urine samples of patients suffering from urinary tract infections and phenotypically identified using the API 20E system (bioMérieux, Marcy l'Etoile, France). Forty-eight isolates were collected from Assiut University hospital and 52 strains from Minia University Hospital.

Sampling process:

Patients were between 25 years old and 70 years old. They suffered from acute uncomplicated UTI. The study patients signed an informed consent form as usually applied before any study procedure was conducted. Midstream urine specimens for bacterial culture were collected in sterile bottles before antibiotic treatment. Samples were incubated aerobically at 37°C for 24 hours on blood agar, Hi-Crome UTI agar, MacConkey agar. Colony counts equal or higher than 10^4 colony forming units (CFU)/mL were considered significant UTI[14]. *E. coli* was preliminarily identified by its characteristic growth on differential media was further confirmed using the API system (API® 20E, bioMérieux, Marcy l'Etoile, France). The study was conducted corresponding to the Declaration of Helsinki and approved by the ethics committee of Assiut University, Faculty of Medicine.

Antibiotic susceptibility testing:

The antibiotic susceptibility profile for *E. coli* isolates was determined using Kirby-Bauer disc diffusion technique[15]. The following commonly used antibiotics were tested: ampicillin, amoxycillin, aztreonam, cefepime, cefotaxime, cefoperazone, ceftazidime, imipenem, meropenem, clindamycin, colistin, gentamicin, amikacin, amoxycillin/clavulanic acid and trimethoprime/sulphamethoxazole. Susceptibility testing was performed by inoculating Mueller-Hinton agar plates (Thermo Fisher Scientific and Waltham, MA, USA) used the suspension equivalent in turbidity to 0.5 McFarland. Then, plates were incubated overnight at 37°C before recording the results. According to the Clinical and Laboratory Standard Institute guidelines, results were interpreted [16].

Colistin susceptibility test:

The use of the disc diffusion method for determination of colistin susceptibility is unreliable which is attributed to the poor and unpredictable diffusion of colistin in the agar [17-22]. Therefore, in our study we used the broth microdilution method was used for the determination of minimal inhibitory concentration to test for colistin susceptibility. Broth microdilution is recommended by both EUCAST as well as CLSI for testing colistin susceptibility. Strains which show colistin minimal inhibitory concentrations (MICs) >2 mg/L were interpreted as resistant according to the EUCAST breakpoints [23].

Detection of *mcr-1* gene of DNA by PCR:

E. coli isolates were screened for detection of *mcr-1* gene which encodes a phosphoethanolamine transferase by PCR. Bacterial DNA templates were obtained using the boiling method from overnight cultures [24]. Briefly, a loopful of pure bacterial colony was suspended in 0.1 ml molecular biology-grade water, boiled for 10 minutes at 100°C, centrifuged for 10 min at 15000 xg for 30 seconds and stored at -20°C. PCR reaction consisted of 12.5 µL MyTaq™ Red Mix (Bioline, London, UK), 5.5 µL of nuclease-free water, 0.5 µL of 10 µM Primers, and 2 µL Bacterial DNA template. PCR conditions were: 1 cycle of initial denaturation at 94 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 90 sec and elongation at 72°C for 60 sec, and a final elongation step at 72 °C for 10 min. Amplified products were visualized by electrophoresis using 1.5% agarose gel electrophoresis. Using the primers CLR F 5'-CGGTCAGTCCGTTTGTTC-3' and CLR R 5'-CTTGGTCGGTCTGTAGGG-3' a 309 bp PCR product should be amplified [3].

RESULTS

Analysis of urine samples:

48 *E. coli* strains were collected from urine samples obtained from Assiut University Hospital, and 52 isolates were obtained from Minia University hospital. The identity of these isolates was confirmed by the API 20E system.

Analysis of antimicrobial resistance:

A total of 13 individual antibiotics and 2 commonly used combined antibiotics were tested (table 1). *E. coli* isolated from Assiut University Hospital showed 100% resistance to ampicillin, amoxicillin, cefotaxime, and ceftazidime. The isolates also showed

high resistance rate against gentamicin (94.4%) and cefepime (89.6%). Similarly, *E. coli* isolated from Minia University Hospital showed 100% resistance to ampicillin, cefotaxime, ceftazidime and cefepime followed by highest resistance to amoxicillin (96.1%) and clindamycin (83.3%). To elucidate the presence of multidrug resistance among isolates, isolates were grouped according to the number of antibiotics to which *E. coli* showed resistance (table 2). It is clear that some strains showed simultaneous resistance to most of the studied antibiotics.

Table 1: Antibiotic resistant pattern of *E. coli* strains isolated from urinary tract

Class of antibiotics		Antibiotic tested		Resistant strains, N (%)		P value
				Assiut University Hospital N=48	Minia University Hospital N=52	(using independent test)
1	β -Lactam	1	Ampicillin	48 (100%)	52 (100%)	1.000 (NS)
2		2	Amoxycillin	48 (100%)	50 (96.1%)	0.510 (NS)
3	Monobactam	3	Aztereonam	35 (72.9%)	36(69.2%)	0.685 (NS)
4	4th generation cephalosporins	4	Cefepime	43 (89.6%)	52 (100%)	0.054 (NS)
5	3rd generation cephalosporins	5	Cefotaxime	48 (100%)	52 (100%)	1.000 (NS)
		6	Cefoperazone	36 (75%)	38 (73.0%)	0.827 (NS)
		7	Ceftazidine	48 (100%)	52 (100%)	1.000 (NS)
6	Carbapenemes	8	Imipenem	17 (35.4%)	19 (36.5%)	0.906 (NS)
		9	Meropenem	17 (35.4%)	19 (36.5%)	0.906 (NS)
7	Polypeptide	10	Clindamycin	33 (68.8%)	45 (83.3%)	0.032 (S)
		11	Colistin	37 (77.1%)	43(82.6%)	0.483 (NS)
8	Aminoglycosides	12	Gentamicin	33 (68.8%)	36 (69.2%)	0.956 (NS)
		13	Amikacin	27 (56.3%)	27 (51.9%)	0.665 (NS)
9	Combination	14	Amoxycillin/clavulanicacid	41 (85.4%)	42 (80.7%)	0.537 (NS)
		15	Trimethoprime/sulphametoxazole	37 (77.1%)	40 (76.9%)	1.000 (NS)

Table 2: Summary of the antibiogram profiles of multidrug resistance strains (MDR) isolated from urine

MDR	Assuit University Hospital N=48		Minia University Hospital N=52	
	Number	Percentage	Number	Percentage
Resistance to 8 antibiotics	0	0%	2	3.8 %
Resistance to 9 antibiotics	7	14.5%	4	7.6 %
Resistance to 10 antibiotics	6	12.5%	6	11.5 %
Resistance to 11 antibiotics	11	22.9%	8	15.3 %
Resistance to 12 antibiotics	14	29.1%	15	28.8 %
Resistance to 13 antibiotics	4	8.3%	9	17.3 %
Resistance to 14 antibiotics	5	10.4%	7	13.4 %
Resistance to 15 antibiotics	1	2.1%	0	0 %

Screening for colistin resistance:

According to the standard guidelines, the broth microdilution technique was used to screen the isolates for colistin resistance. The MICs values ranged from 4 to 16 g/ml. Clinical isolates from both Assiut University Hospital and Minia University Hospital show widespread colistin resistance. Among the 48 strains isolated from Assiut University Hospital, 10 (20.8%) were resistant to colistin while 12 (23.1%) strains isolated from Minia University Hospital were colistin resistant (Table 4). Although the disk diffusion method is not recommended for polymyxins, all *E. coli* isolates carrying the *mcr-1* gene displayed colistin inhibition zones of <11 mm.

Table 3: Rate of colistin resistance among *E. coli* clinical isolates from the different Hospitals

MIC (mg/l)	Assiut University Hospital N=48	Minia University Hospital N=52
< 2	38	40
4	8	10
8	1	1
16	1	1

Detection of *mcr-1* gene encoding phosphoethanolamine transferase by PCR:

Resistant isolates were tested for the presence of *mcr-1* detected by PCR which showed an amplified product of 309 bp (Fig 1). The presence of *mcr-1* gene was detected in all colistin-resistant isolates. Our finding shows the growing widespread of such *mcr-1* gene in both Assuit University Hospital and Minia University Hospital.

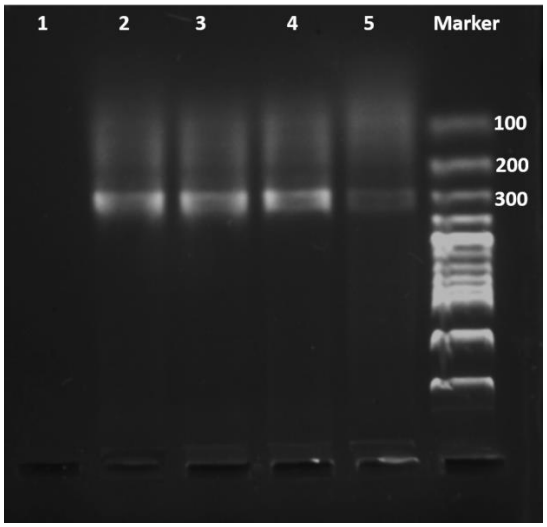

Fig. 1: Agarose gel electrophoresis showing amplification products of the *mcr-1 gene*.
Lane 1: negative control, lines 1, 2, 4, 5: representative clinical isolates showing the amplified product of 309 bp.

DISCUSSION

Antimicrobial resistance in *E. coli* represents an emerging and dangerous public health problem which results in treatment failure [25]. This rise in multidrug resistance of organism is caused mainly by the excessive use of antibiotics by physicians [26,27]. This study provided information about the antimicrobial resistance pattern in *E. coli* strains isolated from patients' urine samples in the Assuit and Minia University Hospital. Generally, resistance patterns detected were similar in both hospitals.

Similar to other studies, our urinary *E. coli* strains from Assiut and Minia university hospitals showed high resistance rates to the commonly used antibiotics [25,27-31]. Moreover, *E. coli* causing UTI are known for their ability to form biofilms cause recurrent leading to persistent and resistant infections [32-34]. Many commonly used antibiotics were completely ineffective against the isolated *E. coli*. Therefore, this study highlights the predominance of MDR in urinary *E. coli*. Besides different isolates showed co-resistance to eight to fifteen antibiotics (table 2).

In this investigation, the highest resistant rates in Assiut and Minia University Hospitals were observed against β-lactam antibiotics. This finding is supported by that of Ranjini, et al., 2015 & Dehbanipour et al., 2016 who recognize excessive use of β -lactam antibiotics in the treatment of UTIs, especially in hospitalized patients, as a possible explanation for the existence of such high resistance rate to these antimicrobial agents [26, 27].

Although imipenem and meropenem showed moderate elevation of resistance in both Assiut University Hospital and Minia University Hospital 17 (35.4%), 19 (36.5%) respectively, they were the most effective drugs. Similar to our findings, other studies mentioned that imipenem and meropenem are active against urinary pathogens [25, 35, 36] . Moreover, our findings reported that *E. coli* isolates exhibited a high degree of co-resistance to other classes of antibacterial included amikacin and trimethoprim-sulfamethoxazole as reported elsewhere [26, 27, 37, 38].

This study reported that colistin resistance to *E. coli* is detected in 10 (20.8%) and 12 (23.1%) strains from Assiut and Minia University Hospitals respectively. In veterinary medicine, colistin is excessively used for different purposes, including prophylaxis and treatment of enteric infections [39] as well as administration with food in poultry farms to prevent infections caused by pathogenic microorganisms [40]. Also, the misuse of colistin antibiotic in agriculture and the poultry industry may be an essential cause of the high progression of *mcr-1* in bacteria from animals and animal products [41]. It was also shown that colistin resistant *E.coli* colonies were identified from pig faecal swabs and chicken[42]. In addition Liu et al.[3] observed that *mcr-1* carriage in *E. coli* isolates that collected from 78 (15%) of 523 specimens of raw meat and 166 (21%) of 804 animals during 2011–14, as well as 16 (1%) of 1322 specimen from inpatients with infection.

Recently, the *mcr-1* gene, which encodes a phosphoethanolamine transferase enzyme, has been described [3]. This gene is plasmid-mediated and codes for an enzyme that belongs to the phosphoethanolamine transferase family. Expression of *mcr-1* in *E.coli* results in the addition of such phosphoethanolamine to lipid A with subsequent resistance to colistin [40].

Since MIC testing is the only recommended method for screening colistin resistance in *Enterobacteriaceae* is the distribution of MIC values of urinary *E. coli* through broth dilution test against colistin were analyzed [39]. Values ranged from 4-16 µg/ml with most samples showed a MIC value of 4 µg/ml in Assuit University Hospital and Minia University Hospital. In addition, the detected *mcr-1* gene in *E. coli* clinical urinary tract strains was confirmed by PCR (figure 1) similarly, *mcr-1* was presented in colistin-resistant *E. coli* isolates from Italy [43]. The actuality that *mcr-1* was detected in all colistin resistant *E. coli* isolates suggests that it is the colistin resistance determinant in our samples. Taken together, our data show that the increased colistin resistance corresponds to *mcr-1* gene detected in *E. coli.* Analysis of the genetic information of the *mcr-1*-positive strains would help us to understand the dissemination as well as the origin of this gene.

This report confirms the alarming prevalence of the plasmid-mediated colistin resistance among Gram-negative bacilli which is coupled with resistance to other antibiotics classes, particularly to the β-lactam drugs. Since *mcr-1* has been found primarily in *E. coli* but has also been identified in other members of the family Enterobacteriaceae in human, animal, and environmental samples, it is recommended to screen other member of Enterobacteriaceae for colistin resistance. A coordinated approach to the prevention of *mcr-1* dissemination is needed to limit the spread of these multidrug-resistant isolates among patients.

CONCLUSION

This study confirmed a high frequency of *E. coli* harboring *mcr-1* genes in clinical urinary isolates from Assuit University Hospital and Minia University Hospital in Egypt. The excessive use or misuse of colistin antibiotic in veterinary medicine and agriculture may be the main cause of the high incidence of *mcr-1* mediated resistance. These strains can cause serious infections resulting in potentially increasing morbidity and mortality. The alarming prevalence of *mcr-1* gene should be treated by continued surveillance and coordinated approach for the prevention of *mcr-1* gene dissemination.

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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