Egyptian Journal of Medical Microbiology Volume 29 / No.1 / January 2020 61-66 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Plasmid mediated colistin resistant genes *mcr-1* and *mcr-2* among *Escherichia coli* and *Klebsiella Pneumoniae* isolates at Zagazig University Hospitals, Egypt

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Key words:
Colistin resistant, PCR, E.coli and k.pneumoniae, mcr-1, mcr-2.

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ABSTRACT

Back ground: *Colistin is considered the last option for severe infections caused by multidrug resistant Gram negative bacteria. The emergence of its resistance constitutes a very serious problem; hence, this study was established.* ***Objectives:*** *This study aims to estimate prevalence of colistin resistance among the clinical isolates of E.coli and k.pneumoniae with detection of the presence of mobilized colistin genes mcr-1 and mcr-2 in those resistant isolates as a possible molecular mechanism for such resistance.* ***Methodology:*** *This cross-sectional study was performed in the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University between January and August 2019. Two hundred isolates of our target organisms were obtained out of 324 specimens from patients admitted at Zagazig University Hospitals. A minimum inhibitory concentration (MIC) of colistin was detected by broth microdilution method. Isolates were reported as resistant if MIC ≥4µg/mL according to CLSI guidelines. PCR for mcr-1 and mcr-2 was done for all colistin resistant isolates.* ***Results:*** *Twenty four (24) isolates resistant to colistin were obtained out of 200 E.coli and k.pneumoniae isolates. Among 24 colistin resistant isolates, we detected mcr-1 gene by PCR in only 2 isolates (8.4%); one E.coli (4.2%) and the other is K.pneumoniae (4.2%) strain. Mcr-2 wasn't detected at all.* ***Conclusion:*** *This study detected the presence of colistin resistance among k.pneumoniae and E.coli isolates from Zagazig University Hospitals by mcr-1 gene but not by mcr-2.*

INTRODUCTION

The worldwide spread of antibiotic resistance is a challenging issue as it badly affects the patient clinical and financial conditions in healthcare settings. This is more obvious among bacteria of Gram negative appearance especially the *Enterobacteriaceae*, which constitutes an alarming problem as the management choices available for multidrug resistant bacteria are limited[1, 2].

The emergence of extended spectrum β-lactamases (ESBLs), *Klebsiella pneumoniae* carbapenemases and New Delhi metallo-beta-lactamase (NDM) together with the increased use of carbapenem with subsequent emergence and widespread of its resistance, made colistin the last option for treatment of these infections[3, 4].

Colistin is a cyclic polycationic peptide. It interacts with the negatively charged lipopolysaccharide in the outer membrane (LPS) causing its disruption with increase in the outer membrane permeability and subsequently cell death [5, 6].

Colistin resistance was traditionally mediated by mutations causing modifications in the Lipid A molecule or even its complete loss[3], until the emergence of plasmid mediated resistance which was first reported in Nov. 2015 and was mediated by the *mcr-1* gene which was detected in *E.coli* from pigs in China[7, 8].

Then due to a transposon carrying *mcr-1* from plasmids and subsequent movement to different plasmids and bacterial strains, colistin resistance became popular all over the world [9].

Additional plasmid mediated genes were then identified; *mcr-2* and *mcr-3* were also detected sharing some nucleotide similarity with *mcr-1*[7]. Then genes up to *mcr-8* were discovered in 2018[6].

Moreover, it was found that *mcr-1* gene occurred with other genes of resistance as ESBL and NDM leading to fatal bacterial infections which would be difficult to be cured[9].

Hence, our aim of work was designed to investigate the occurrence of colistin resistance among *E.coli* and *K.pneumoniae* isolates through *mcr-1* and *mcr-2* genes.

METHODOLOGY

This cross-sectional study was performed in the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University and was approved by the Institutional Review Board (IRB),

Faculty of Medicine, Zagazig University, Egypt. Informed consents were obtained from all patients.

Bacterial isolates:

A total 200 *E.coli* and *K.pneumoniae* strains were obtained from 324 clinical specimens which were isolated from urinary catheters, blood, sputum and surgical wound of patients admitted at Zagazig University Hospitals during the period from January to August 2019. Samples were examined by Gram stain, cultivated on MacConkey, Blood, and CLED (only for urine) (Oxoid, UK) and incubated at 37°C for 24 hours aerobically. Gram negative, lactose fermenting colonies were further identified using standard biochemical tests and confirmed by API 20 E (Bio-Merieux. France).

Phenotypic detection of antibiotic sensitivity:

Disk diffusion Kirby– Bauer method:

On Mueller–Hinton agar (Oxoid, UK) plates according to the guidelines of Clinical and Laboratory Standards Institute[10]. The antibiotic disks tested were ceftazidime (30µg), cefotaxime (30µg), cefepime (30µg), imipenem (10µg), meropenem (10µg), gentamicin (10µg), amikacin (30µg), ciprofloxacin (5µg), levofloxacin (5µg), sulfamethoxazole/ trimethoprim (1.25/23.75µg), piperacillin/tazobactam (100/10µg), doxycycline (30µg) and nitrofurantoin (300 µg) for urine specimens only (Oxoid, UK)**;** colistin was excluded due to poor diffusion of the large colistin molecule[11]. The phenotype of *Enterobacteriaceae* was defined as MDR (resistant to ≥1 antimicrobial agent in ≥3 antimicrobial classes) and XDR (non susceptible to one agent or more in all but ≤ 2 antimicrobial classes which means that the bacterial isolate remains susceptible to only one or two classes)[12].

Broth microdilution method: A minimum inhibitory concentration (MIC) of colistin was detected and isolates were reported resistant if MIC was ≥4 µg/Ml [10].

Molecular detection of colistin resistance:

Using QIAamp® DNA Mini kit (Qiagen, Germany), DNA was extracted from the isolated strains. Amplification was performed using a set of primers (iNtRON Biotechnology, Korea) as listed in Table 1. The amplification procedure was performed according to the following program: " at 94 °C: initial denaturation for 5 min and 25 cycles of denaturation for 1 min, then annealing for *mcr-1* at 51°C and 53 °C for *mcr-2* for 30 s, finally at 72 °C: extension for 30 s and a final extension for 5 min"[5]. Lastly, the amplification products were analyzed by electrophoresis and compared with suitable DNA ladder.

Table1: Primer sequences of *mcr-1* and *mcr-2* genes[13].

Gene	Sequences (5'→ 3')	Amplicon size (bp)
*mcr-1*F *mcr-1*R	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTA GGG	309 bp
*mcr-2*F *mcr-2*R	TGGTACAGCCCCTTTATT GCTTGAGATTGGGTTATGA	1,747 bp

Statistical analysis:

To analyze collected data, Statistical packages (EPI-info Version 6.04 and SPSS Version 20 inc. Chicago, USA) were used. To compare proportions, we used Chi-square test. P Values < 0.05 were considered to be statistically significant at 95% confidence interval.

RESULTS

Out of 324 collected specimens, 200 isolates of our target organisms were obtained with an isolation rate of 61.7 % including 107 *E.coli* (53.5%) and 93 *K.pneumoniae* isolates (46.5%).

Table 2 demonstrates distribution of our isolates among the different hospital wards where the highest rate was from ICUs with 46% isolation rate and the lowest was from pediatric wards with isolation rate of 9.5% which was statistically significant* (p=0.005).

Table 2: Distribution of *E. coli* and *K. pneumoniae* among different hospital wards

Ward	No. (%) of isolates		Total	X^2	P
	E.coli	*K.Pneumoniae*			
ICUs	40 (20%)	52 (26%)	92 (46%)	14.8	0.005*
Internal medicine	21 (10.5%)	7 (3.5%)	28 (14%)		
Surgery	11 (5.5%)	14 (7%)	25 (12.5%)		
Oncology unit	20 (10%)	16 (8%)	36 (18%)		
Pediatric wards	15 (7.5%)	4 (2%)	19 (9.5%)		
Total	107 (53.5%)	93 (46.5%)	200 (100%)		

*p < 0.05 is significant. ICU, intensive care unit.

Basing on the results of antibiotic susceptibility tests, 73% of our isolates were MDR and 27% were XDR. Most *E.coli* and *K.pneumoniae* isolates showed high resistance to cefepime (92.9%, 90.1%) and ceftazidime (78.6%, 93.1%) respectively. However, the most susceptibility was attributed to doxycycline (37.5%, 34.1%) and gentamycin (39.2%, 50%) among our *E.coli* and *K.pneumoniae* isolates respectively. Patterns of antibiotics resistance of our isolates have been designed in Figure 1.

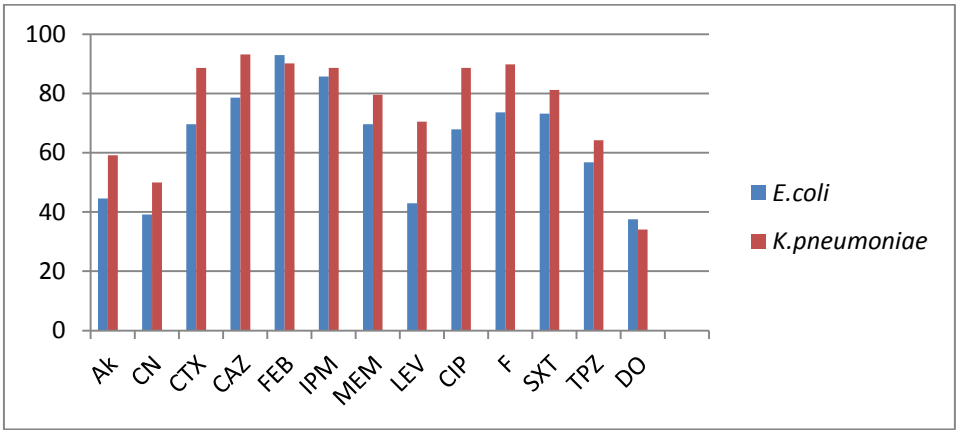

Fig (1): Patterns of antimicrobial resistance among our isolates

Out of 200 isolates, 12% (n=24) were reported as resistant to colistin using tube microdilution method. Among these resistant isolates, 66.7% (n=16) were *K. pneumoniae* and 33.3% (n=8) were *E.coli* and this was statistically significant (p=0.03) (table 3). Our isolates prevalence in various specimens is illustrated in Table 4. Urine (n=9) with 37.5% and wound (n=4) with 16.7% showed the highest and lowest rates of colistin resistance, respectively among all collected specimens with no statistically significant association with colistin susceptibility (p=0.18) (Table 4).

Table 3: Distribution of colistin susceptibility between both isolates

Isolate	Resistant N=24	Susceptible N=176	Total N=200	X^2	P
E.coli	8 (33.3%)	99 (56.2%)	107 (53.5%)	4.45	0.03*
K.pneumoniae	16 (66.7%)	77 (43.8%)	93 (46.5%)		

p<0.05 significant.

Table 4: Distribution of colistin Susceptibility among sources and isolated strains

Specimen	Resistant (N=24)		Total No. (%)	Susceptible (N=176)		Total No. (%)
	E.coli No. (%)	*K.pneumoniae* No. (%)		*E.coli* No. (%)	*K.pneumoniae* No. (%)	
Urine	4 (16.7%)	5(20.8%)	9 (37.5%)	58 (32.9%)	26 (14.8%)	84 (47.7%)
Blood	3 (12.5%)	3(12.5%)	6 (25%)	22 (12.5%)	23 (13.1%)	45 (25.6%)
Sputum	-	5(20.8%)	5 (20.8%)	-	13 (7.4%)	13 (7.4%)
Wound	1 (4.1%)	3(12.5%)	4 (16.7%)	19 (10.8%)	15 (8.5%)	34 (19.3%)
Total	8 (33.3%)	16 (66.7%)	24 (100%)	99 (56.2%)	77 (43.8%)	176 (100%)
X^2	4.79					
P	0.187					

p>0.05 non-significant.

Mcr-1 gene of colistin resistance was detected by PCR in 2 of the resistant isolates (8.4%) figure (2). On contrary, *mcr-2* not detected at all.

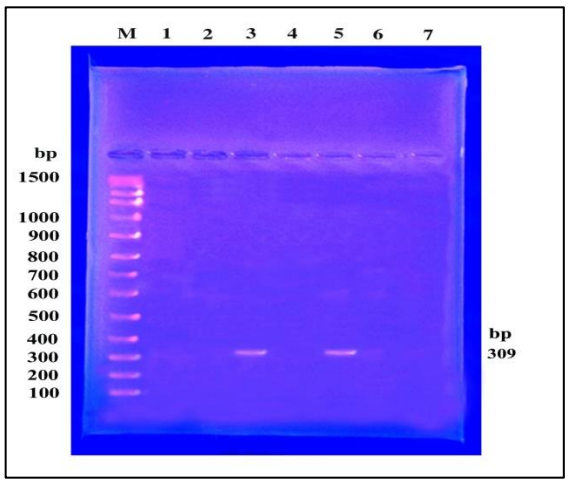

Fig. 2: PCR amplification of *mcr-1* gene; lane (M) show 100Bp Mwt marker, lane (3, 5) show two positive results of *mcr-1* gene.

DISCUSSION

The occurrence of colistin resistance among *Enterobacteriaceae* is a global problem which may be attributed to its wide use for carbapenem resistant isolates[14,2]. In addition, some countries use this antibiotic as additive for animal food to increase its quality [15].

Plasmid mediated colistin resistance which can be transferred between Gram negative bacteria is considered a dangerous problem in spreading antibiotic resistance making study of prevalence of colistin resistance very important to limit its spread[7].

In our study, the rate of *E.coli* and *K.pneumoniae* isolation was 61.7% which comes in agreement with the rates reported by ML and Raja[16] (61.9%) and Kaur and his colleagues[17] found out of 276 gram negative isolates, *E.coli* and *K.pneumoniae* of a rate 65.6%, in addition, Emara and his colleagues [18] declared 72.5% isolation rate for *E.coli* and *K.pneumoniae*.

Among various hospital wards, The prevalence of our isolates was highest from ICUs with 46% isolation rate and the lowest was from pediatric wards with isolation rate of 9.5% and this was statistically significant (p=0.005). These results were not in agreement with that obtained by Moosavian and Emam[13] who found that outpatient clinic and infectious ward had the highest and lowest rates respectively 54.9% and 0.4%. This divergence in results may be due to difference in type of samples, number of cases and compliance with the infection control measures.

Our isolated strains had marked resistance to fourth generation cephalosporines cefepime (92.9%, 90.1%) and third generation cephalosporines ceftazidime (78.6%, 93.1%) for *E.coli* and *K. pneumoniae* isolates respectively. And resistance to carbapenem antibiotics (80%). This is co matched with the results reported previously in Egypt by Zaki and his colleagues[19] who found their isolated strains had resistance to cefepime (78%), ceftazidime (60%) and cefotaxime (56%). Around 50% of their isolates had resistance to carbapenem antibiotics.

Less resistance was noticed to doxycycline (37.5%, 34.1%) and gentamycin (39.2%, 50%) among our *E.coli* and *K.pneumoniae* isolates respectively. This comes in contrary to the results of Rapoport and his colleagues[20] and Buchler and his colleagues[21] who reported poor sensitivity to gentamycin (20%, 15%) respectively. This susceptibility variation may be attributed to the variation in antibiotics regimens in different geographical regions.

Regarding phenotypic susceptibility of colistin, we detected 24 isolates (12%) as colistin resistant by tube microdilution method. this was in accordance with the results obtained by Moosavian and Emam[13] who detected colistin resistance in 13.6%, meanwhile Luo and his colleagues[15] and Buchler and his colleagues[21] found colistin resistance in a rate of 3% and 3.8% respectively.

Among our colistin-resistant isolates, 66.7% were *K. pneumoniae* and 33.3% were *E.coli* which was statistically significant (p=0.03). This was nearly similar to which declared by Zaki and his colleagues[19] who detected colistin resistance of a rate 44% *in K.pneumoniae* and 42% in *E.coli*.

On the other hand, Emara and his colleagues[18] found among their colistin resistant isolates, 80% were *K. pneumonia*, only 10% *E. coli* and 10% *P. aeruginosa*. Also, Moosavian and Emam[13] who detected colistin resistance in 59.4% of *E.coli* and 40.6% of *K.pneumoniae*. This mismatch may be due to difference in sample size.

In our study, The most common sources of colistin resistant isolates were urine catheters with a rate of 37.5% then blood with 25% then sputum with 20.8% and the least rate was from wounds with 16.7% and this was statistically non significant (p=0.18)

Similar result was obtained by Zaki and his colleagues[19] who declared that most isolates were from urine 46% then blood 30% and wounds 24%. Also Moosavian and Emam[13] reported that urine specimens were the commonest source of isolation with a rate of 87,4%.

As regard the genotypic results of colistin resistance, *mcr-1* was detected by PCR in 2 isolates (8.4%); one *E.coli* isolate (4.2%), the other *K.pneumoniae* isolate (4.2%). In contrast, other studies found the gene in

lower rates as Moosavian and Emam[13] detected *mcr-1* in *E.coli* isolates with a rate of 1.2% and in *K. pneumoniae* isolates (0.4%) and Zaki and his colleagues[19] detected *mcr-1* gene in 2 out of 50 (4%) colistin resistance. Meanwhile, Luo *and* and his colleagues[15] found the gene in 21 colistin resistance *E.coli* out of 40 (52.5%) and explained their higher rates of *mcr-1* carriage due to the high amount of livestock and meat in China, where prevalence of colistin-resistant isolates is high.

Moreover, Emara and his colleagues[18] and Tanfous and his colleagues[22] reported that colistin *mcr-1* gene was not detected among their phenotypically resistant isolates. This discrepancy was best explained by (WHO, 2018)[23] which reported that negative results in the PCR cannot be used to predict susceptibility to colistin, because the test cannot exclude the presence of chromosomal mechanisms of resistance or even of novel *mcr* genes that are not included in the test**.**

Finally, none of our isolates harbored *mcr-2* gene and this goes hand with hand with the results reported by Zaki and his colleagues[19] and Luo and his colleagues[15]. The *mcr-2* gene was only reported in Belgium by Sun and his colleagues[24] which posed a hypothesis that *mcr-2* dissemination occurs by a different mechanism.

CONCLUSION

This study highlighted the emergence of colistin resistance through *mcr-1* gene among *E.coli* and *K.pneumoniae* while *mcr-2* was not confirmed.

Recommendations

We recommend further studies with larger sample size and broader spectrum of Gram negative bacteria for accurate detection and follow up this serious problem. Moreover, strict application of infection control and antibiotic policies to control spread of antibiotic resistance.

Acknowledgement

We would like to introduce our deep gratitude to our colleagues at different Departments in Zagazig University Hospitals for their cooperation to collect our specimens.

Limitations: None

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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