Egyptian Journal of Medical Microbiology Volume 28 / No.4 / October 2019 87-93 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Colistin Susceptibility and the Effect of Colistin-sulfadiazine Combination among Multidrug Resistant *E. coli* and *K. pneumoniae* at Egyptian Intensive Care Units

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Key words:
Antibiotic stewardship; antibiogram; middle income countries; Enterobacteriaceae; infection control

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ABSTRACT

Background: *Colistin is the last resort for treatment of infections caused by multidrug-resistant Gram-negative bacilli.* ***Objectives:*** *This study aimed to evaluate colistin susceptibility among multidrug resistant E. coli and K. pneumoniae isolates, to determine the prevalence of mcr-1& mcr-2 genes carrying isolates and to evaluate the synergistic activity of colistin in combination with the sulfadiazine against colistin-resistant isolates.* ***Methodology:*** *1632 E. coli and K. pneumoniae isolates were collected and identified. Antibiotic susceptibility& MIC for colistin were detected followed by investigating for mcr-1 and mcr-2 genes by PCR. The effect of colistin–sulfadiazine combination was tested by E-test/ agar diffusion method.* ***Results:*** *Among 1218 MDR E. coli and K. pneumoniae isolates, 34 (2.79 %) colistin resistant isolates were detected. The isolates were most frequently isolated from urine 10 (29.4%) and tracheal aspirate 5 (14.7%). Highest resistance rates were reported: ceftazidime (97.1%), cefepime (94.1%), and imipenem (82.3%). Least resistance rates were displayed for amikacin and sulfamethoxazole/trimethoprim (73.5%), and tobramycin (70.5%) respectively. The mcr-1 gene was detected in only one E. coli strain while the mcr-2 gene was not detected. By using colistin-sufadiazine combination, a decrease in colistin MIC ≥ 2 dilutions was observed for 26/34 isolates (76.5%) including the single mcr-1 positive isolate.* ***Conclusion:*** *The spread of colistin resistance threatens its use as the last resort for MDR E. coli and K. pneumoniae. The presence of mcr-1 alarms facing pan-drug resistance among Enterobacteriaceae. To its limit spread, a great concern should be paid to continuous surveillance, putting into practice an effective antibiotic stewardship program and enhanced infection control measures. The use of colistin/sulfadiazine combination represents an alternate and could be used for some cases of colistin-resistant Gram negative bacilli.*

INTRODUCTION

The emergence and spread of carbapenem-resistant Enterobacteriaceae (CRE), has led to the introduction of colistin, the only left antibiotic choice for the treatment of CRE. Colistin is a polycationic molecule. It acts by interaction with the outer membrane of the lipopolysaccharide of bacterial cell membrane by displacing divalent cations from the negatively-charged phosphate group of lipid A ending in cell lysis [1].

For many years, mechanisms of colistin resistance were attributed to chromosomal mutations maintained in the presence of colistin selection and excessive use of colistin. However, they are not transferred to other organisms. More recently, a novel plasmid-mediated colistin resistance genes (*mcr-1*, *mcr-2*) were detected worldwide. *Mcr-1* gene encodes for a phosphoethanolamine transferase enzyme that results in transferring a phosphoethanolamine to lipid A leading to resistance to colistin. *Mcr-2* gene was also identified to be associated with colistin resistance. This type of resistance is not related to the use of colistin, but the plasmid can be transferred between different strains and species by conjugation and transformation [2]. Following these important discoveries, several international teams started looking for those genes in the existing collections of bacteria [1-4]. In 2018, Caselli and his colleagues [5] reported that *mcr-1* containing isolates can be detected from environmental surfaces with high frequency, indicating that this plasmid has the ability to spread among human pathogens. This emphasizes that surveillance of colistin resistance mechanisms present in a certain population is a vital process for the effective treatment of bacterial infections and monitoring the development and spread of resistance [6].

Colistin was increasingly prescribed as the last resort treatment for infections caused by MDR bacteria [7]. Hereby, the use of combination therapies to control bacterial infections appeared as a rescuer. Recently it

has been demonstrated that sulphonamide compounds (e.g. sulfadiazine, trimethoprim, sulfamethoxazole, trimethoprim-sulfamethoxazole) have a synergistic activity when combined with colistin [8]. This may give some hope to restore the antibacterial activity of colistin.

Many clinical microbiology laboratories in Egypt do not routinely test colistin against *E. coli* and *K. pneumonia*, which makes it difficult to retrieve the resistance rates from local data. Thus the current study was conducted with the aim to evaluate colistin susceptibility pattern among multidrug resistant *E. coli* and *K. pneumoniae* isolates, to determine the prevalence of *mcr-1* and *mcr-2* genes carrying isolates and to evaluate the synergistic activity of colistin in combination with the sulfadiazine against colistin-resistant isolates.

METHODOLOGY

Study settings and design:

A cross sectional study was conducted at Zagazig University Hospitals which comprise nine specialized hospitals; serve populations from the delta, Sinai and eastern provinces of Egypt. Clinical isolates of *E. coli* and *K. pneumoniae* were collected from intensive care units (ICUs) admitted patients over one year; from June 2017 to May 2018. The study was approved by the Institutional Review Board (IRB) (no. 4821) - Faculty of Medicine, Zagazig University, Egypt. An informed written consent was obtained from each patient or the guardians of unconscious patients.

Bacterial identification and antibiotic susceptibility testing of isolated bacteria:

It was done by Vitek 2 system (Biomerieux, Marcy l'Etoile, France). It included the following antibiotics: imipenem, meropenem, amikacin, tobramycin, piperacillin/ tazobactam, ceftazidime, cefepime, ciprofloxacin, levofloxacin and sulfamethoxazole/trimethoprim. The results were interpreted according to CLSI recommendations 9. *Escherichia coli* (*E. coli*) ATCC 25922 was used as quality control strains (American Type Culture Collection Global Bioresource Center, Manassas, VA, USA). Isolates that were non-susceptible to at least one agent in three or more classes of antimicrobials were considered as multi drug resistant (MDR) isolates 10.

Detection of MIC for colistin:

For isolates that showed colistin resistance by Vitek 2 system, MIC values of colistin were detected by broth microdilution method. An improved calcium-enhanced Muller-Hinton (CE-MH) medium was used for colistin susceptibility testing 11. Colistin sulfate powder (Livzon pharmaceutical group, China) was used for broth microdilution. European Committee on Antimicrobial Susceptibility Testing breakpoints were used for the interpretation of colistin MICs, isolates were reported as colistin resistant if MIC was > 2 ug/ml 12. Sub-cultures of colistin resistant subset was done on glycerol broth and then stored at -20°C. *E. coli* ATCC®25922 was used as a quality control strain (American Type Culture Collection Global Bioresource Center, Manassas, VA, USA).

Detection of *mcr-1* and *mcr-2* genes:

The isolates that exhibited MIC values for colistin > 2 ug/ml were further investigated for the existence of *mcr-1* and *mcr-2* genes. Plasmid was extracted from the clinical isolates by QIAprep® Spin Miniprep kit (Qiagen GmbH, Hilden, Germany). The used primers were: For *mcr1*; F:5′CGGTCAGTCCGTTTGTTC3′ - R:5/-CTTGGTCGGTCTGTAGGG-/3. For *mcr-2*; F:5′TGGTACAGCCCCTTTATT 3′- R:5′GCTTGAGATTGGGTTATGA 3′ The amplification cycle: 5 min at 94°C, followed by 30 cycles of 45 s at 94°C, 1 min at 60°C (for *mcr-1*) or 1 min at 55°C (for *mcr-2*), 1 min at 72°C, and a final extension time of 7 min at 72°C 1. Electrophoresis was performed with gel 2% for 20 minutes. The products were visualized by UV and compared with DNA ladder.

Colistin–sulfadiazine combination by E-test / agar dilution method:

All colistin-resistant isolates were included (including isolates positive for *mcr-1* gene) and one colistin-susceptible clinical isolate of *E. coli* was used as control.

Agar plates containing sulfadiazine were prepared as previously reported 13 with modifications. Briefly, freshly prepared sulfadiazine (sulfadiazine powder supplied from Sigma-Aldrich) was added to the medium preparation of Muller-Hinton (MH) agar to obtain the corresponding final concentrations of 8 μg/ml of sulfadiazine. The cooled medium was then poured into sterile petri dishes. A suspension of bacteria from an overnight culture was prepared in distilled water to obtain a final concentration of approximately 10^8 CFU/ml (0.5 McFarland). After swabbing in three directions, colistin E-test strips were placed, and after 24 hours of incubation at 37˚C, the colistin MICs were read 8.

Statistical analysis:

The Statistical Package for the Social Sciences (SPSS), version 22 (SPSS Inc., Chicago, IL, USA) was used for data coding, validation and analysis. Frequencies and proportions were used to present the data.

RESULTS

Overall, 1632 non-duplicate clinical isolates of *E. coli* and *K. pneumoniae* were collected from different types of clinical specimens over the study period. Susceptibility testing revealed 1218 MDR isolates. Thirty-four colistin-resistant isolates (19 *Escherichia coli* and 15 *Klebsiella pneumoniae* strains) were

detected by broth microdilution method. The prevalence rate of colistin resistance among the MDR isolates was 2.79 %.

For colistin-resistant isolates: The most common sources as regards MDR *E. coli* isolates were urine 10 (52.6%) and tracheal aspirate 4 (21.1%). The most common sources as regards MDR *K. pneumonia* were tracheal aspirate 5 (33.3%) (Fig 1).

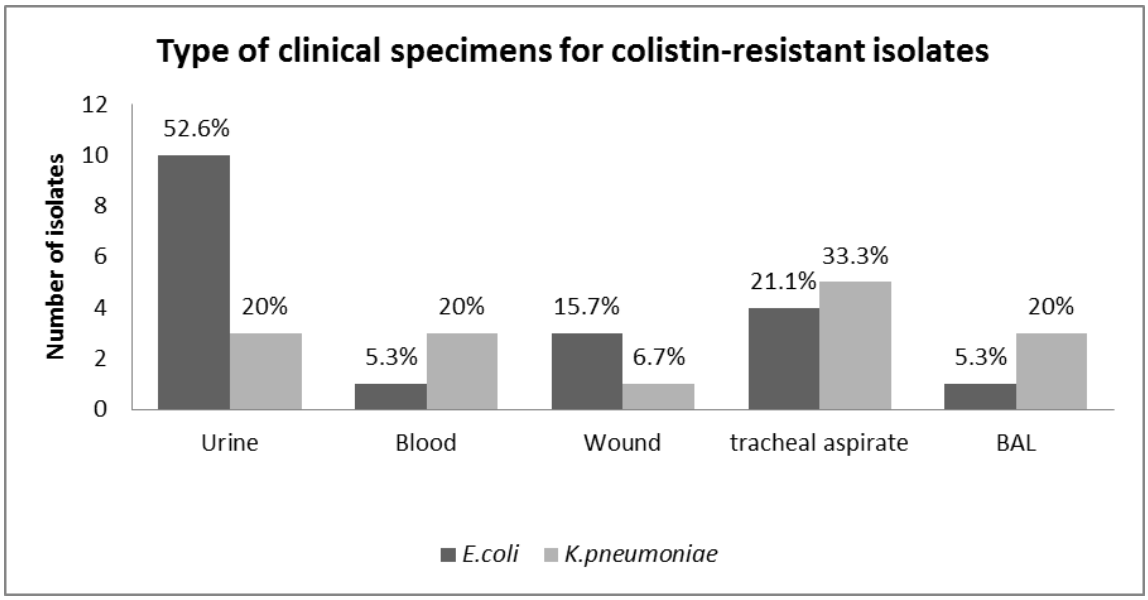

Fig. 1: For colistin-resistant *E. coli* isolates; 10 (52.6%) isolates were from urine, 1 (5.3%) isolate (was) from blood, 3 (15.7%) isolates were from wound, 4 (21.1%) isolates were from tracheal aspirate and 1 (5.3%) isolate was from bronchoalveolar lavage (BAL). For colistin-resistant *K. pneumoniae* isolates; 3 (20%) isolates were from urine, 3 (20%) isolates were from blood, 1 (6.7%) isolate was from wound, 5 (33.3%) isolates were from tracheal aspirate and 3 (20%) isolates was from BAL.
BAL: bronchoalveolar lavage

Colistin MIC was found to be 4 ug/ml in 12 isolates (35.3%), 8 ug/ml in 13 isolates (38.2%), 16 ug/ml in 7 isolates (20.6%) and 32 ug/ml in 2 isolates (5.9%), (Table 1). The results of antibiotic susceptibility among these isolates showed elevated resistance levels to the majority of the tested antibiotic classes expressing the MDR or XDR phenotypes. The isolates had the greatest resistance to the third generation cephalosporine, ceftazidime (97.1%) and fourth generation cephalosporine, cefepime (94.1%). Resistance to carbapenem antibiotics imipenem and meropenem were 82.3% and 76.5% respectively. Resistance to piperacillin-tazobactam was 82.3%. Resistance to quinolone antibiotics ciprofloxacin and levofloxacin were 91.1% and 85.2% respectively. Least resistance rates were for amikacin (73.5%), tobramycin (70.5%) and sulfamethoxazole/trimethoprim (73.5%).

Table 1: Colistin MIC of colistin-resistant isolates

Colistin MIC	*E. coli* No. (%)	*k. pneumoniae* No. (%)	Total No. (%)
4 ug/ml	8 (42.1)	4 (26.7)	12 (35.3)
8 ug/ml	6 (31.6)	7 (46.7)	13 (38.2)
16 ug/ml	4 (21.1)	3 (20)	7 (20.6)
32 ug/ml	1 (5.3)	1 (6.6)	2 (5.9)
Total	19 (100)	15 (100)	34 (100)

Colistin resistance genes were detected by PCR. *Mcr-1* was detected in only one *E. coli* isolates but not detected in *K. pneumoniae* isolates; representing 5.3% of *E. coli* isolates and 2.9 % of colistin-resistant isolates. *Mcr-2* was not detected at all. The presence of *mcr-1* gene was found to be associated with high colistin MIC (32 ug/ml), (Table 2). This only *mcr-1* positive *E. coli* isolate was obtained from urine of a male patient with urinary tract infection at the emergency department. This isolate was sensitive to meropenem and amikacin but resistant to other antibiotics.

Table (2): Features of colistin-resistant isolates

E. coli	**Sample**	**Colistin MIC (ug/ml)**		***mcr-1* gene**	***mcr-2* gene**
		Before combination	**After combination**		
1	urine	8	2	negative	negative
2	blood	4	4	negative	negative
3	urine	8	1	negative	negative
4	wound	16	4	negative	negative
5	TA	8	1	negative	negative
6	urine	32	8	**Positive**	negative
7	BAL	16	2	negative	negative
8	urine	8	8	negative	negative
9	TA	8	1	negative	negative
10	urine	8	4	negative	negative
11	urine	16	2	negative	negative
12	urine	4	4	negative	negative
13	wound	8	2	negative	negative
14	urine	16	8	negative	negative
15	TA	4	0.5	negative	negative
16	TA	4	1	negative	negative
17	urine	8	1	negative	negative
18	wound	4	1	negative	negative
19	urine	4	0.5	negative	negative
K. pneumoniae	**Sample**	**Colistin MIC (ug/ml)**		***mcr-1* gene**	***mcr-2* gene**
		Before combination	**After combination**		
1	TA	16	2	negative	negative
2	blood	8	8	negative	negative
3	TA	4	0.5	negative	negative
4	BAL	8	8	negative	negative
5	BAL	16	4	negative	negative
6	wound	8	2	negative	negative
7	TA	32	4	negative	negative
8	urine	8	2	negative	negative
9	blood	4	4	negative	negative
10	urine	8	2	negative	negative
11	BAL	8	1	negative	negative
12	TA	16	2	negative	negative
13	blood	4	0.5	negative	negative
14	TA	8	1	negative	negative
15	urine	4	1	negative	negative

BAL: bronchoalveolar lavage TA: tracheal aspirate

In colistin – sulfadiazine combination test, 34 colistin-resistant isolates were included (19 *E. coli* isolates, included only one positive for *mcr-1* gene, and 15 *K. pneumoniae* isolates). One colistin-susceptible clinical isolate of *E. coli* was used as control.

The synergistic effect was obtained when FIC (The fractional inhibitory concentration index) ≤ 0.5 with a decrease ≥ 2 dilutions for colistin MIC. A decrease in colistin MIC ≥ 2 dilutions was observed for 26/34 isolates (76.5%) including *mcr-1* positive *E. coli* isolate. The other eight isolates (5 *E. coli* and 3 *K. pneumonia*) showed either no change in colistin MIC or either only one-fold decrease in colistin MIC (Table 3).

Table (3): the result of colistin-sulfadiazine combination by determining the number of colistin MIC two-fold dilutions, ≥ 2 dilutions or < 2 dilutions

Number of isolates	**Colistin + Sulfadiazine combination**		
	No change	**< 2 dilutions**	**≥ 2 dilutions**
E. coli (19 isolates)	3	2	14
K. pneumoniae (15 isolates)	3	0	12
Total (34 isolates)	6	2	26 (76.5%)

DISCUSSION

Colistin resistance signals the breach of polymyxins, one of the last-resort antibiotics for the multidrug-resistant Gram-negative bacteria 1. Colistin resistance follows the increasing trend in consumption of colistin in human medicine, especially in countries with high rates of CRE 14, including Egypt 3, 15.

Prevalence data on colistin resistance are overall scarce 16. The current study reports 2.79 % prevalence rate for colistin resistance. Similar reports were published earlier (0.5%) [17,18]. The relatively lower rate in those studies could be attributed to the methodological differences between the automated systems used for colistin MIC evaluation. EUCAST highly recommends the use of broth microdilution for determining the correct colistin MIC value, especially when it ranges from 1 to 2 ug/ml with subsequent underestimation of colistin resistance 16.

Antibiotic susceptibility testing of the 34 colistin resistant isolates showed high levels of resistance to different classes of antibiotics. Comparable results were reported earlier from Mansoura City, Egypt 1; India 19; Italy 20; Spain 21; Vietnam 22; France 23. This emphasizes the association between colistin resistance and resistance to carbapenems, cephalosporins, quinolones, aminoglycosides and β-lactams. In the meantime, we reported higher resistance rates as regards amikacin and sulfamethoxazole-methotrexate than Zaki *et al*., 1, but it aligns with another study from Latin America 24.

In May 2016, the first report of *mrc-1* in a clinical isolate from Egypt was released 3. They isolated one *E. coli* isolate out of 241 clinical isolates. In April 2018, Zaki *et al*., 1 reported the isolation of two *mcr-1* containing isolates; one *E. coli* and one *K. pneumonia* out of 50 colistin resistant Enterobacteriaceae isolates, but *mcr-2* was not detected. To the best of our knowledge, no other Egyptian researchers studied the prevalence of *mcr-1* and *mcr-2* in clinical isolates. In the current study, *mcr-1* gene was detected in only one *E. coli* strain with a prevalence of 1/34 (2.9%). On the other hand, *mcr-2* gene was not detected in any of the investigated isolates.

Variable frequencies were revealed from different countries, in Arabian Peninsula only four *mcr-1* positive *E. coli* isolates were found among 75 colistin resistant Enterobacteriaceae strains 4, in the Sultanate of Oman only one *E. coli* strain was found to carry *mcr-1* gene out of 22 studied isolates but there was no strain carrying *mcr-2* gene 25. In Hong Kong five *mcr-1* positive isolates were detected from 62 colistin resistant isolates 26. Also, in North Italy, the *mcr-1* was detected in 26 *E. coli* isolates among 90 colistin resistant isolates 6. However, in South Africa, the *mcr-1* gene was detected in 83% of colistin resistant isolates which were very high 2. This variability may be due to the difference in the pattern of colistin use among livestock in different countries; the worldwide high prevalence of *mcr-1* in animal isolates, versus that of human clinical isolates, advocates animals and their products as potential sources of *mcr-1* in humans 3.

Mcr-1 is co-localized with many types of resistance genes which differ according to the type /sequence of plasmid itself 4. This may lead to the variability of the resistance pattern of *mcr-1* containing isolates among different studies. An evident finding in the current study is that the only *mcr-1* positive *E. coli* isolate was resistant to all tested antibiotics except for meropenem and amikacin. This coincides with previous reports 3.

The *mcr-1* carrying plasmids can be stably maintained and more rapidly disseminate under the selective pressure imposed by the use of antimicrobial agents other than colistin 6. Thus, the difference in the application of infection control measures and implementation of antibiotic stewardship programs may be responsible for the variation in the susceptibility reports from different healthcare settings. Hospitals must be aware of this new threat. Clinical microbiology laboratories should consider testing for colistin susceptibility more frequently, for example in situations involving multidrug-resistant Gram-negative bacteria. Of note that the disk diffusion is not a reliable test for colistin susceptibility, rather a method of measuring the minimum inhibitory concentration should be used 27.

In this study, the colistin in combination with sulfadiazine showed synergistic activity for 26/34 isolates (76.5%) including *mcr-1* positive *E. coli* isolate. Okdah et al. 8 reported that a decrease in colistin MIC ≥ 2 dilutions was observed for 27/30 strains (90%), and only 3 strains showed no change in colistin MIC, also he reported that the combination of colistin and sulfadiazine possessed the best synergistic and bactericidal activity. These enhanced colistin-based combinations are of clinical interest, given the failure of colistin monotherapy and the emergence and increase in both chromosomal and plasmidic colistin resistance 28. The synergistic effect of colistin-based combinations is based on the ability to disturb the bacterial cell wall's the outer membrane 2. This support the hypothesis that colistin facilitates the entry of sulfadiazine into the bacterial cell by destabilizing the bacterial cell wall. Even if the combination does not work on all colistin-resistant isolates, yet it represents an alternate to be implemented for specific cases.

CONCLUSION

This study confirms the continuous spread of the plasmid mediated gene *mcr-1*. It alarms facing pan-drug resistance in the family of Enterobacteriaceae. Though *mcr-1* was detected in a low prevalence with the absence of *mcr-2*, yet the presence of *mcr-1* gene in our province should not be passed over. Great concern

should be paid to continuous surveillance, and improving prevalence data. Enhanced infection control precautions should be considered when suspecting colistin resistance. A more rational use of antibiotics is vital. Implementation of antibiotic stewardship program has to be set as a first priority for healthcare authorities. Exploration of the knowledge, practice and attitude of healthcare workers towards such a problem is highly recommended so as planning for suitable health education programs could take place, this will help in combating such threatening condition. The study supports the use of colistin/sulfadiazine combination against colistin resistant Gram-negative bacteria.

Limitations of the study

We assessed the presence of the *mcr-1* and *mcr-2* genes in the phenotypically colistin resistant isolates only. It is possible, although this is unlikely, that few *mcr-1* and *mcr-2* positive isolates may have been missed by restricting testing to the colistin resistant subset. Being the first study at the investigated hospital to explore *mcr* genes, we didn't investigate other *mcr* genes; *mcr-3*, *mcr-5* and *mcr-5*. They are the subject of further studies by the authors.

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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