Evaluation of Polymyxin NP Test as a Rapid Method for Detection of Polymyxins Resistance in Enterobacteriaceae

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Key words:
Polymyxin NP test; Polymyxin resistance; Colistin resistance; Broth microdilution (BMD); Multidrug resistance (MDR).

Background and study aim: Polymyxins are considered the last remedy for multidrug-resistant *Enterobacteriaceae*. However, due to their overuse, Polymyxin resistance rose and posed the urge to find new methods to aid their rapid diagnosis. So, this study aimed to assess the diagnostic efficacy of the rapid polymyxin NP test for diagnosis of colistin and polymyxin-resistant Enterobacteriaceae and detect the frequency of mcr-1 and mcr-2 genes among the MDR-*Enterobacteriaceae* isolates.

Patients and Methods: This study included 42 isolates of MDR-Enterobacteriaceae that were subjected to broth microdilution method to detect the MICs for polymyxin B and colistin, rapid polymyxin NP test using Polymyxin B, and conventional PCR for the identification of Polymyxins resistant genes; mcr-1 and mcr-2.

Results: Eighteen out of 42 isolates were resistant to colistin (42.9%), while 15/42 were resistant to polymyxin (35.7%). For both colistin and polymyxin MIC, the polymyxin NP test exhibited an overall sensitivity, specificity, PPV, and NPV of (77.8%, 91.7%, 87.5%, 84.6%) and (93.3%, 92.6%, 87.5%, 96.2%) respectively. Seventeen isolates were positive for mcr-1 gene (40.5%) and five isolates were positive for mcr-2 gene (11.9%).

Conclusion: The rapid polymyxin NP test is an easy and quick that can reliably detect both polymyxin-resistant and susceptible *Enterobacteriaceae* isolates. This test can be implemented as a screening tool in the outbreak management and active surveillance for presence of Polymyxins resistance. Therefore, combined with PCR, it can play a substantial role to contain antimicrobial resistance and prevent transfer of resistance genes among patients.

INTRODUCTION

The carbapenemase-producing *Enterobacteriaceae* (CPE) are among the most clinically significant multidrug-resistant bacteria (MDR). Owing to the increase in infection with MDR bacteria, along with sparsely available antibiotic options, polymyxin B and colistin usage has risen again after being halted in the 1970s. Despite their potential toxicity, polymyxins gained worldwide attention as CPE are still susceptible to this old class of antimicrobials **[1-3]**.

This mounting colistin usage can be the origin of the acquisition of *Enterobacteriaceae* of colistin resistance in addition to the carbapenem resistance **[4]**. In research from the USA, 13% of carbapenem-resistant *Klebsiella pneumoniae* isolates were resistant to colistin **[5]**.

Lipopolysaccharide modification by adding either or both phospho-ethanolamine and 4-amino-l-arabinose cationic groups to lipopolysaccharide can give rise to colistin acquired resistance in *Enterobacteriaceae* through reducing polymyxin binding to the bacterial outer membrane. This can be associated with chromosome encoded mechanisms **[6]**. Other researchers reported that resistance might be plasmid-mediated through the mcr-1 gene **[7-10]**.

Though broth microdilution (BMD) is the standard method for testing the

susceptibility to polymyxins, it is meticulous and needs a long time (24 hours). Other techniques such as disk diffusion and E-test have been proposed for performing polymyxin antimicrobial susceptibility. They can also give results in 18–24 hours. But due to polymyxin molecules' poor diffusion in agar, these tests yielded high false susceptibility results (up to 32%) **[11, 12]**.

To overcome these drawbacks, rapid two-hour assays were developed using the principle of detecting acidic products of bacterial metabolism in the existence of colistin or polymyxin B **[13-16]**.

Rapid Polymyxin NP test was introduced for quickly detecting colistin-resistant *Enterobacteriaceae.* The basis of this test is detecting the colour change caused by rapid glucose metabolization by the growing bacteria in certain concertation of colistin or Polymyxin B **[14]**. Despite being sensitive and specific, these assays do not supply data on the resistance mechanisms **[17]**.

So, the current study aimed to assess the diagnostic efficacy of the rapid polymyxin NP test using Polymyxin B for the diagnosis of colistin- and polymyxin resistant *Enterobacteriaceae* and determine the frequency of mcr-1 and mcr-2 genes among the colistin-resistant *Enterobacteriaceae* at Ain Shams University hospitals (ASUH).

MATERIAL AND METHODS

Bacterial strains:

This prospective study included 42 isolates of MDR-*Enterobacteriaceae* collected from different clinical samples of 42 patients submitted for routine culture and sensitivity in main microbiology laboratory, ASUH. This work was conducted during the period from April 2020 to August 2020.

Study procedures:

All *Enterobacteriaceae* isolates were preliminary identified by conventional microbiological techniques including colonial morphology, gram stain characteristics and biochemical reactions. Final identification was confirmed by Vitek2C system (Biomerieux, France).

Multidrug-resistant *Enterobacteriaceae* isolates were chosen to be resistant to one or more agents in at least three groups of antibiotics by disc diffusion method as per **CLSI** breakpoints **[18]**. Broth microdilution method (BMD) was performed to detect the minimum inhibitory concentration (MIC) for polymyxin B and colistin according to **EUCAST** breakpoints **[19]**. Subculture was performed for all isolates on Mueller Hinton agar. Then, Rapid polymyxin NP test was done **[14]**.

Conventional Polymerase chain reaction (PCR) was done for the identification of Polymyxins resistant genes; mcr-1 (forward primer: AGTCCGTTTGTTCTTGTGGC, reverse primer: AGATCCTTGGTCTCGGCTTG) and mcr-2 (forward primer: CAAGTGTGTTGGTCGCA GTT, reverse primer: TCTAGCCCGACAAGCA TACC) genes **[8]**.

Antibiotic susceptibility testing by broth microdilution method (BMD):

The standard BMD was performed and interpreted as per **EUCAST [19]** to determine the polymyxin B and colistin MICs using cation-adjusted Mueller-Hinton broth (MHB-CA), Colistin sulphate and polymyxin B powder (Sigma-Aldrich Chemical Co.). Stock solutions for both Colistin sulphate and Polymyxin B sulphate were prepared at a concentration of (50mg/ml) from which a fresh working solution was prepared to reach a final concentration of 64 ug/ml. *Escherichia coli* ATCC 25922 was used as a negative control for susceptibility testing.

Polymyxin NP test:

We carried out Polymyxin NP test using Polymyxin B antibiotic according to the experimental procedure explained by Nordmann et al. **[14]**. To prepare solutions for the polymyxin NP test, we used the following reagents: Polymyxin B powders, Phenol red powder, MHB-CA, 10% HCl solution, and anhydrous glucose. We purchased all reagents from (Sigma-Aldrich Chemical Co.). After preparation of the reagents, we inoculated the 96-well polystyrene micro-test plates with a freshly prepared 3.0 McFarland bacterial suspension. Then, we incubated the inoculated tray for up to 4 hours at 35 ± 2°C in ambient air without sealing and inspected it visually after 10 min and then every hour for 4 hours. We used *E. coli* ATCC 25922 as a Polymyxin-susceptible control and *Proteus mirabilis* ATCC 12453 as Polymyxin-resistant isolates.

Conventional Polymerase chain reaction (conventional PCR):

We examined all the 42 *Enterobacteriaceae* isolates enrolled in the current study for the presence of mcr-1 and mcr-2 genes using conventional PCR **[8]**.

DNA extraction, purification, and detection

DNA extraction and purification using GeneJET PCR Purification Kit (Qiagen, USA) was performed as per the manufacturer's instructions. For DNA amplification and detection, we used a master mix consisting of Thermo Scientific DreamTaq Hot Start Green DNA Polymerase (Qiagen, USA**),** primers of mcr-1 (forward primer: AGTCCGTTTGTTCTTGTGGC, reverse primer: AGATCCTTGGTCTCGGCTTG) and mcr-2 (forward primer: CAAGTGTGTTGGTCGC AGTT, reverse primer: TCTAGCCCGACAAGCA TACC) genes. PCR was done using a thermal cycler with the following thermal cycling conditions: 95°C for 3 minutes, then 40 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 minute, and finally 72°C for 15 minutes.

We used *E. coli* ATCC 25922 as a negative control and *Proteus mirabilis* ATCC 12453 as a positive control. The quality control reference strains were supplied by (Remmel, UK).

We used gel electrophoresis and DNA-binding dye to visualize the results of PCR reaction. A standard, or DNA ladder, was included so that the size of the fragments in the PCR sample can be determined. For mcr-1 gene, the PCR reaction produced a 320-base pair (bp) and for mcr-2 gene produced a 715-base pair (bp) **[20]**.

Statistical Analysis:

The collected data were processed using Statistical Package for Social Science **(SPSS 20).** For descriptive statistics, we calculated frequency and percentage of non-numerical data. As for analytical statistics, Chi-Square test was used to examine the relationship between two qualitative variables. Fisher's exact test was used when the expected count is less than 5 in more than 20% of cells. We calculated Sensitivity, Specificity, Positive predictive value (PPV), Negative predictive value (NPV), and accuracy to detect the diagnostic performance. Major errors and very major errors were calculated. P value was used to indicate levels of significance where $P \geq 0.05$ = non-significant (NS), $P < 0.05$ = significant (S), and $P < 0.01$ = highly significant (HS).

RESULTS

In this study, 42 well identified MDR-*Enterobacteriaceae* isolates (36 isolates of *Klebsiella* (85.7%) and 6 isolates of *E.coli* (14.3%)) were randomly obtained from patients' specimens during the period from April 2020 to the end of August 2020.

Demographic data:

Regarding the demographic data, we found that the age of the patients enrolled in the current study ranged from 9 days to 77 years old and inter quartile range (IQR) is 14 (0.75 - 48). The number of female and male patients included were equal (21/42, 50%). Concerning the sample type, the majority of our isolates were recovered from blood cultures (12/42, 28.6%), followed by urine cultures (10/42, 23.8%), skin and soft tissue infections (9/42, 21.4%), sputum (7/42, 16.7%), central line (3/42, 7.1%), and finally CSF culture (1/42, 2.4%).

Antibiotics intake:

Among the 42 patients included in our study, we found that 30 (71.4%) patients received antibiotics. Meropenem rated the first among all the used antibiotics where 10/30 (33.3%) patients received it as shown in table (1).

Antimicrobial susceptibility testing (AST):

As for the antibiotic susceptibility testing, although not all isolates included in our study were tested for all antibiotics, yet, all of those tested recorded 100% resistance rate against almost all β-lactams with exception of ceftazidime (97.6%) and imipenem (90.0%). The results of the rest of the antibiotics are shown in table (2).

Results of Colistin MIC:

As for colistin MIC results, they ranged between 0.5 to 32 ug/ml. 18 isolates out of 42 were resistant to colistin (42.9%). The majority of resistant isolates were *Klebsiella* isolates (83.3%) in comparison with *E. coli* (16.7 %). Regarding the MIC 50 and MIC 90, they were (2 and 16) respectively (table 3).

Results of Polymyxin B MIC:

For polymyxin B MIC, the results ranged between 0.5 to 16 ug/ml. 15 isolates out of 42 were resistant to polymyxin B (35.7%) The majority of resistant isolates were *Klebsiella* isolates (86.7%) *E. coli* was (13.3%). MIC 50

and MIC 90 were (2 and 16) respectively (table 4).

Results of Polymyxin NP test:

Among the 42 tested isolates, 26 isolates were sensitive (26/42, 61.9%), including 22 *Klebsiella* isolates (22/26, 84.6%), and four *E. coli* isolates (4/26, 15.3%). While among the sixteen resistant isolates, fourteen *Klebsiella* isolates (14/16, 87.5%) and two *E. coli* isolates (2/16, 12.5%) were resistant (table 5, figure 1).

Results of PCR:

Among the 42 tested isolates for mcr-1 gene by PCR, 17 isolates were positive (17/42, 40.5%) out of them 14 *Klebsiella* (14/36, 38.9%) and 3 *E. coli* (3/6, 50%) (table 6, figure 2).

While 5 isolates were positive for mcr-2 gene, 11.9% (5/42) all of them were *Klebsiella* (5/36, 13.9%) (table 7, figure 3).

Correlation between Phenotypic methods and MIC for both Colistin and Polymyxin B:

The correlation between MIC method for colistin versus polymyxin B MIC, Polymyxin NP test and PCR for mcr-1 and mcr-2 genes, was highly statistically significant as shown in table (8).

Similarly, the correlation between the MIC method for polymyxin B versus colistin MIC, Polymyxin NP test and PCR for mcr-1 and mcr-2 genes, was highly statistically significant ($P<0.01$) as shown in table (9).

The diagnostic performance and evaluation of Polymyxin NP test:

The diagnostic performance of Polymyxin NP test compared to MIC for colistin and polymyxin B was summarized in tables (10) and (11) respectively.

Table (1): The percentage of antibiotic intake among the 30 studied patients who received antibiotics in the current study.

Antibiotics taken	**No. (%)**
Penicillins	**7 /30 (23.3%)**
Penicillin	1/30 (3.3%)
Amoxicillin/ Clavulanic acid	2/30 (6.7%)
Ampicillin/ Sulbactam	4/30 (13.3%)
Cephalosporin	**11/30 (36.7%)**
Ceftriaxone	1/30 (3.3%)
Cefepime	2/30 (6.7%)
Ceftazidime	2/30 (6.7%)
Cefoperazone	5/30 (16.7%)
Cefotaxime	1/30 (3.3%)
Carbapenem	**12/30 (40%)**
Meropenem	10/30 (33.3%)
Imipenem/ Cilastatin	2/30 (6.7%)
Aminoglycosides	**8/30 (26.7%)**
Gentamicin	1/30 (3.3%)
Amikacin	7/30 (23.4%)
Quinolones	**7/30 (23.3%)**
Ciprofloxacin	3/30 (10%)
Levofloxacin	4/30 (13.3%)
Others	**13/30 (43.3%)**
Linezolid	2/30 (6.7%)
Vancomycin	5/30 (16.6%)
Clindamycin	4/30 (13.3%)
Metronidazole	2/30 (6.7%)

Table (2): Antimicrobial susceptibility testing by Disc Diffusion Method and Vitek2 C for 42 studied isolates in the current study.

Antibiotic sensitivity by Vitek2C and disc diffusion method	No.	Sensitive	Resistant	Intermediate-Sensitive
		No. (%)	No. (%)	No. (%)
Ampicillin	10	0 (0.0%)	10 (100.0%)	0 (0.0%)
Ampicillin/Sulbactam	10	0 (0.0%)	10 (100.0%)	0 (0.0%)
Amoxicillin/Clavulanate	32	0 (0.0%)	32 (100.0%)	0 (0.0%)
Piperacillin/Tazobactam	11	0 (0.0%)	11 (100.0%)	0 (0.0%)
Cefoxitin	9	0 (0.0%)	9 (100.0%)	0 (0.0%)
Cefotaxime	32	0 (0.0%)	32 (100.0%)	0 (0.0%)
Ceftazidime	42	1 (2.4%)	41 (97.6%)	0 (0.0%)
Cefoperazone	27	0 (0.0%)	27 (100.0%)	0 (0.0%)
Ceftriaxone	36	0 (0.0%)	36 (100.0%)	0 (0.0%)
Cefpodoxime	5	0 (0.0%)	5 (100.0%)	0 (0.0%)
Cefepime	14	0 (0.0%)	14 (100.0%)	0 (0.0%)
Clofazimine	1	0 (0.0%)	1 (100.0%)	0 (0.0%)
Imipenem	10	1 (10.0%)	9 (90.0%)	0 (0.0%)
Meropenem	18	0 (0.0%)	18 (100.0%)	0 (0.0%)
Amikacin	40	9 (22.5%)	27 (67.5%)	4 (10.0%)
Gentamycin	11	2 (18.2%)	8 (72.7%)	1 (9.1%)
Tobramycin	10	0 (0.0%)	10 (100.0%)	0 (0.0%)
Ciprofloxacin	19	0 (0.0%)	18 (94.7%)	1 (5.3%)
Levofloxacin	32	4 (12.5%)	26 (81.3%)	2 (6.3%)
Doxycycline	28	6 (21.4%)	22 (78.6%)	0 (0.0%)
Sulfamethoxazole/ trimethoprim	41	4 (9.8%)	37 (90.2%)	0 (0.0%)
Nitrofurantoin	9	2 (22.2%)	7 (77.8%)	0 (0.0%)

Table (3): Colistin MIC results among the 42 studied isolates in the current study.

		No. = 42
Colistin MIC (ug/ml)	Median (IQR)*	2 (1 - 8)
	Range	0.5 – 32
	Percentile 90	16
	0.5	6 (14.3%)
	1	13 (31.0%)
	2	5 (11.9%)
	4	3 (7.1%)
	8	8 (19.0%)
	16	6 (14.3%)
	32	1 (2.4%)

***IQR: Interquartile range.**

Table (4): Polymyxin B MIC results among the 42 studied isolates in the current study.

		No. = 42
Polymyxin B MIC (ug/ml)	Median (IQR)	2 (1 - 4)
	Range	0.5 – 16
	Percentile 90	16
	0.5	8 (19.0%)
	1	12 (28.6%)
	2	7 (16.7%)
	4	6 (14.3%)
	8	4 (9.5%)
	16	5 (11.9%)

***IQR: Interquartile range.**

Table (5): Results of Polymyxin NP test for the 42 isolates included in the current study.

Organism	Polymyxin NP test			
	Positive (resistant) No= 16	%	Negative (sensitive) No= 26	%
Klebsiella	14	87.5 %	22	84.6 %
E coli	2	12.5 %	4	15.4 %

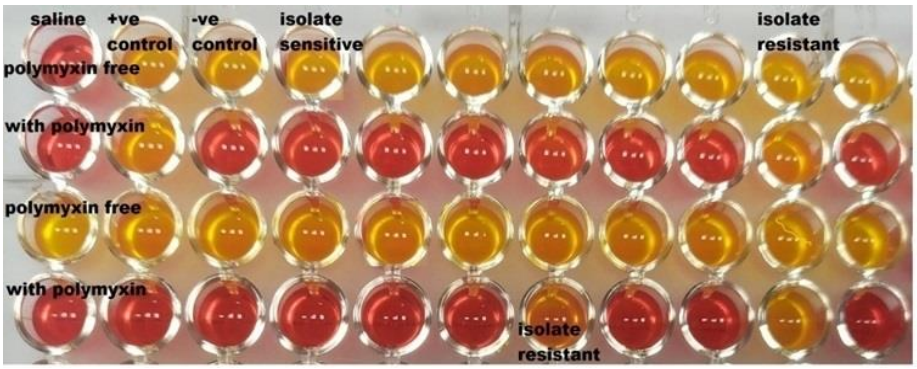

Figure (1): Polymyxin NP test in a Microtitre plate show first and 3rd row contain polymyxin free solution, the 2nd row contains polymyxin NP solution. first well in the first and 2nd row were inoculated with saline, the 2nd well in the first and 2nd rows were inoculated by positive control strain, first and 2nd rows were inoculated with saline, the 3rd wells in the first and 2nd rows were inoculated by negative control strain, the 4th wells in the first and 2nd rows were inoculated by tested isolate and so on. color change from red to yellow or red to orange indicate resistant bacterial growth and positive polymyxin NP test.

Table (6): Distribution of mcr-1 gene among 42 tested isolates in the current study.

		Klebsiella		*E. coli*		Total	
		No	%	No	%	No	%
mcr-1	Negative	22	61.1%	3	50%	25	59.5%
	Positive	14	38.9%	3	50%	17	40.5%
	Total	36	100%	6	100%	42	100%

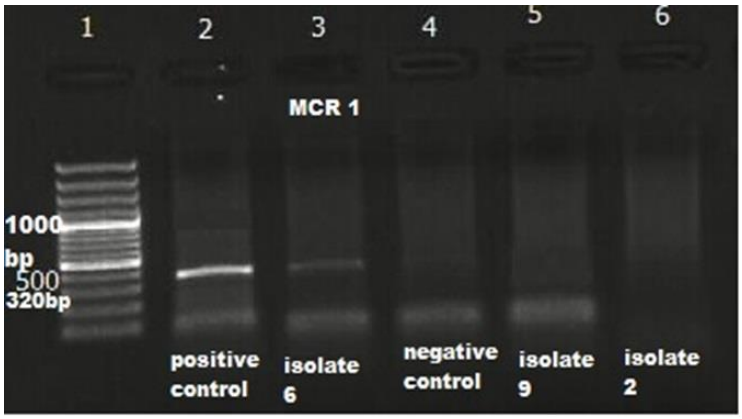

Figure (2**):** mcr-1 PCR results on gel electrophoresis. DNA ladder in lane 1, positive control for mcr-1 gene at 320 bp in lane 2, positive isolate in lane 3, negative control in lane 4 and negative isolates in lanes 5 and 6.

Table (7): Distribution of mcr-2 gene among different tested isolates in the current study.

		Klebsiella		*E. coli*		Total	
		No	%	No	%	No	%
mcr-2	Negative	31	86.1%	6	100%	37	88.1%
	Positive	5	13.9%	0	0%	5	11.9%
	Total	36	100%	6	100%	42	100%

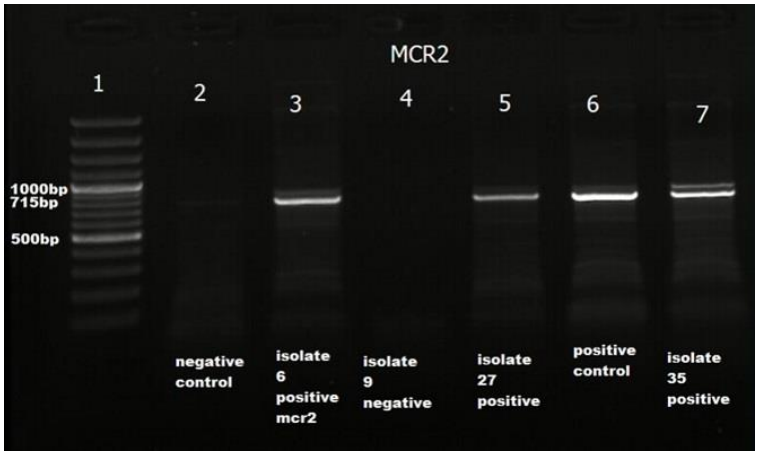

Figure (3**):** mcr-2 PCR results on gel electrophoresis. DNA ladder in lane 1, negative control in lane 2, positive isolate in lanes 3, 5, 7, positive control for mcr-2 gene at 715 bp in lane 5 and negative isolates in lane 4.

Table (8): Correlation between colistin MIC versus polymyxin B MIC, polymyxin NP test and PCR for mcr-1, 2 genes among 42 tested isolates in the current study

		Colistin MIC interpretation		Test value	P-value	Sig.
		Sensitive	Resistant			
		No. = 24	No. = 18			
Polymyxin B MIC interpretation	Sensitive	24 (100.0%)	3 (16.7%)	31.111*	0.000	HS
	Resistant	0 (0.0%)	15 (83.3%)			
Polymyxin NP test	Sensitive	22 (91.7%)	4 (22.2%)	21.034*	0.000	HS
	Resistant	2 (8.3%)	14 (77.8%)			
PCR of mcr-1 gene	Negative	24 (100.0%)	1 (5.6%)	38.080*	0.000	HS
	Positive	0 (0.0%)	17 (94.4%)			
PCR of mcr-2 gene	Negative	24 (100.0%)	13 (72.2%)	7.568*	0.006	HS
	Positive	0 (0.0%)	5 (27.8%)			

P≥0.05 = non-significant (NS), P<0.05 = significant (S), and P<0.01 = highly significant (HS)
*** Chi-square test**

Table (9): Correlation between polymyxin B MIC versus colistin MIC, polymyxin NP test and PCR for mcr-1, 2 genes among 42 tested isolates in the current study.

		Polymyxin B MIC interpretation		Test value	P-value	Sig.
		Sensitive	Resistant			
		No. = 27	No. = 15			
Colistin MIC interpretation	Sensitive	24 (88.9%)	0 (0.0%)	31.111*	0.000	HS
	Resistant	3 (11.1%)	15 (100.0%)			
Polymyxin NP test	Sensitive	25 (92.6%)	1 (6.7%)	30.190*	0.000	HS
	Resistant	2 (7.4%)	14 (93.3%)			
PCR of mcr-1 gene	Negative	25 (92.6%)	0 (0.0%)	34.314*	0.000	HS
	Positive	2 (7.4%)	15 (100.0%)			
PCR of mcr-2 gene	Negative	27 (100.0%)	10 (66.7%)	10.216*	0.001	HS
	Positive	0 (0.0%)	5 (33.3%)			

P≥0.05 = non-significant (NS), P<0.05 = significant (S), and P<0.01 = highly significant (HS)
* Chi-square test

Table (10): Diagnostic performance of Polymyxin NP test compared to the reference method MIC for colistin among 42 tested isolates in the current study.

	TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV	Accuracy	Major errors (%)	Very major errors (%)
Polymyxin NP test	14	22	2	4	77.8%	91.7%	87.5%	84.6%	0.857	2 (8.3%)	4 (22.2%)

TP: True positive, TN: True negative, FP: False positive, FN: False negative, PPV: Positive predictive value, NPV: Negative predictive value.

Table (11): Diagnostic performance of Polymyxin NP test compared to the reference method MIC for polymyxin B among 42 tested isolates in the current study.

	TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV	Accuracy	Major errors (%)	Very major errors (%)
Polymyxin NP test	14	25	2	1	93.3%	92.6%	87.5%	96.2%	0.929	1 (4.1%)	2 (11.1%)

TP: True positive, TN: True negative, FP: False positive, FN: False negative, PPV: Positive predictive value, NPV: Negative predictive value.

DISCUSSION

The carbapenemase-producing *Enterobacteriaceae* are among the most clinically significant MDR bacteria. Such a rise drove an increase in the usage of polymyxins because of limited effective alternative antibiotic treatment. So, this global concern of polymyxins and the mounting use of colistin explicates the addition of acquired colistin resistance to the resistant traits in *Enterobacteriaceae* **[3]**.

We aimed to assess the diagnostic efficacy of the rapid polymyxin NP test using Polymyxin B for the diagnosis of colistin- and polymyxin resistant *Enterobacteriaceae* and determine the frequency of mcr-1 and mcr-2 genes among the colistin-resistant *Enterobacteriaceae* at ASUH.

In the present study, the age of the patients ranged from 9 days to 77 years. The number of female and male patients included were equal (21/42, 50%). Concerning the sample type, it was observed that majority of isolates were retrieved from blood cultures (12/42, 28.6%).

In a similar pattern to our study, research conducted in Croatia reported that their patients harboring colistin-resistant isolates were equally distributed between both sexes (50% each), and their ages ranged from 6 to 84 years. The predominant specimen were blood cultures (9/24), followed by urine samples (5/24) **[21]**.

As for the antibiotic susceptibility testing, all the tested isolates recorded a 100% resistance rate against almost all β-lactams with exception of ceftazidime (97.6%) and imipenem (90.0%). These findings came in concordance with the internationally high level of antimicrobial resistance among all classes of antimicrobial, especially in Egypt which caused surging in colistin use as the last resort antimicrobial.

In Egypt, a one-year retrospective study showed that Gram-negative isolates displayed a high rate of resistance to all used antibiotic classes. This study reported that resistance towards the Beta-lactam group ranged from 70% to more than 98% against different antibiotic members of this group **[22]**. Such a high resistance was demonstrated by other researchers as well. In Croatia, the sixteen enterobacterial isolates were resistant to almost all antibiotics; β-lactams, ciprofloxacin, and gentamicin. Except for colistin, the MIC90 of all antibiotics exceeded 128 mg/L **[21]**.

Regarding antimicrobial consumption among the 42 patients included in our study, we found that 30 (71.4%) patients received antibiotics. Meropenem rated the first where 10/30 (33.3%) of our patients received it.

Other researchers reported a high overall antibiotic consumption among different hospitals. They found that Cephalosporins were the most usually prescribed antibiotics, followed by quinolones,

penicillins, and carbapenems. They reported high significant pearson's correlation coefficient factors (r) between carbapenem consumption and its resistance rate of 0.271 and 0.427 for *E. coli* and *Klebsiella pneumoniae* respectively **[23]**.

Also, other investigators demonstrated that the rate of antibiotic usage among their patients was very high ranging from 32.9% to 91.7% and 59% of them were receiving one or more antibiotics. Third-generation cephalosporins ranked first among the prescribed antibiotics and accounted for 28.7%. Penicillins with beta-lactamase inhibitors and metronidazole derivatives accounted for 19.7% and 15.2% respectively **[24]**.

Unlike our study, other researches in China and Egypt proved that cephalosporins are the most received drug **[23, 24]**. Whereas in the current study, Meropenem was the most frequently used one. This backs to the high level of multidrug resistance that was detected in the last years and obliged the physicians to prescribe carbapenem as the last option before resorting to polymyxins.

For colistin MIC results, our results ranged between 0.5 to 32 ug/ml. 18 isolates out of 42 were resistant to colistin (42.9%). The majority of resistant isolates were *Klebsiella* isolates (83.3%) in comparison to *E. coli* (16.7 %).

Similarly, a study conducted in Thailand reported that colistin resistance was identified in 13 (35%) *E. coli* and 213 (76%) *Klebsiella pneumonia* isolates, with MIC ranging from 0.5 to 32 mg/L and 0.25 to >128 mg/L respectively **[25]**.

In an Indian study, they found that among their 138 isolates, meropenem, colistin, and double resistance were detected in 110, 31, and 21 isolates, respectively. Noticeably, 90.5% (19/21) of the dual colistin-resistant and carbapenem-resistant isolates were *Klebsiella pneumoniae* **[26]**.

For polymyxin MIC done for our tested isolates, the results ranged between 0.5 to 16 ug/ml. Fifteen isolates out of 42 were resistant to polymyxin (35.7%). The majority of the resistant isolates were *Klebsiella* isolates (86.7%) and *E. coli* was (13.3%).

A Chinese study reported a lower resistance among 504 carbapenem-resistant *Enterobacteriaceae* isolates. Only 19 isolates exhibited resistance to polymyxin B, with MICs ranging from 4 μg/mL to ≥256 μg/mL **[27]**.

Other researchers found that out of 110 evaluated isolates, 51 isolates were polymyxin-resistant (MIC ranging from 4 to > 64 μg/mL) and 59 were polymyxin-susceptible (MICs ranging from ≤ 0.125 to 2 μg/mL) **[28]**.

In light of results stated worldwide, we found that they agreed to a great extent with those reported in our study and this affirms the fact of increasing resistance against colistin and polymyxin B which are considered the last hope for treatment of MDR organisms, this foreshadows an upcoming worldwide disaster.

As regards the polymyxin NP test, the overall sensitivity, specificity, PPV, and NPV versus the colistin MIC BMD were 77.8%, 91.7%, 87.5%, and 84.6% respectively. While, versus the polymyxin MIC BMD, the overall sensitivity, specificity, PPV, and NPV were 93.3%, 92.6%, 87.5%, and 96.2% respectively.

Our results are in concordance with those reported by many authors where their studies showed excellent sensitivity and specificity [14, 29-32]. Other authors from Greece found that the Rapid Polymyxin NP test yielded a high sensitivity (99%) and a lower specificity of 82%. Although their results were different from ours, yet, these findings still point out that the Rapid Polymyxin NP test can be used as a screening test for early diagnosis of colistin-resistant isolates **[33]**. Thus, this test can represent a reliable alternative to BMD, especially in resource-limited settings.

Although the sensitivity of the test obtained in our study was lower for colistin MIC compared with the polymyxin MIC, many worldwide studies stated that the Rapid Polymyxins NP test exhibited an outstanding performance for detecting resistant and susceptible *Enterobacteriaceae* for both polymyxin B and colistin. We proved in our study that this test is easy to perform and could be accomplished within 4 hours compared to the MIC by the BMD method, which consumes around 24 hours.

A Brazilian study asserted this fact and reported that the required time to obtain the result was 2 hours for the Rapid Polymyxins NP test versus 24 hours for the BMD. In addition, this test is more advantageous, being less burdensome. This fast identification of polymyxin-resistant bacteria might aid in precisely identifying the optimum therapy choices, and rapidly implementing

contact precaution measures, thus halting further outbreaks with MDR-isolates **[29]**.

In our study, we carried out conventional PCR for confirmation of the presence of polymyxins resistant genes (mcr-1, 2 genes). In correlation with Colistin MIC, we found that 17/18 colistin-resistant isolates had the mcr-1 gene (94.4%) and five (27.8%) isolates harbored the mcr-2 gene. Whilst, correlating it with polymyxin MIC revealed that all the 15 (100%) polymyxin-resistant isolates were positive for the mcr-1 gene and 5/15 (33.3%) positive for the mcr-2 gene. We observed that the mcr-1 gene was found in all of the 14 Rapid Polymyxins NP positive isolates, while, the mcr-2 gene was detected solely in five isolates.

Although in our study mcr-1 and mcr-2 genes were present in most of our isolates, this represented a hindrance to finding a statistical relationship between the types of genes and the results of the rapid polymyxin NP test.

Research conducted in Switzerland found that the Rapid Polymyxins NP displayed 100% sensitivity when performed using polymyxin-resistant isolates of environmental, animal, and human origin and mediated by the plasmid carrying genes mcr-1 or mcr-2 **[16]**.

On the other hand, investigators from Brazil reported that Polymyxins NP gave false-positive results in two polymyxins-susceptible isolates of their 19 selected isolates (11 polymyxins-resistant and 8 polymyxins-susceptible) carrying the mcr-1 gene **[29]**.

A study in Thailand detected the mcr-1 gene in 11/37 *E. coli* isolates and their colistin MIC range was 4–32 mg/L, while, they found the mcr-1 gene in 4/280 *Klebsiella pneumoniae* isolates with a colistin MIC range of 4–64 mg/L **[34]**.

The world has been suffering for a long time from the era that we fear the most; the era of antimicrobial resistance, this dreadful social challenge. Nowadays, the globe is attempting to find a way out of this trouble. But, since we are unable to add new therapeutic agents to our inventory, so, the only possible solution we have is to try decreasing the rate of colonization and infections with resistant strains, thereby reducing mortality and morbidity. To do so, we need to apply preemptive measures before we are left handcuffed without any antimicrobial choices.

In the current study, the rapid Polymyxin NP test proved to be a reliable, easy, affordable, and speedy assay to perform. Moreover, it exhibited promising performance compared to our reference technique. Hence, we can deduce that this test can be implemented as a screening tool in the outbreak management and active surveillance for the presence of Polymyxins resistance. Therefore, combined with PCR, it can play a substantial role as a part of infection control measures to contain antimicrobial resistance and prevent the transfer of resistance genes among patients.

CONCLUSION

The rapid polymyxin NP test is an easy, quick, sensitive, and specific test that can reliably detect both polymyxin-resistant and susceptible Enterobacteriaceae isolates, whatever the molecular mechanism of resistance. Even more, it is less sophisticated and can provide results at least 16 hours before the BMD method .

The performance of the polymyxin NP test in the current study was lower for colistin MIC in comparison with the polymyxin B MIC .

Therefore, we recommend using the polymyxin NP test for early detection of polymyxin-resistant Enterobacteriaceae, especially in low resource health care settings. Further studies on broad scales with large sample sizes are recommended to study the effect of each mcr gene separately on the sensitivity and specificity of this test.

ACKNOWLEDGMENT: None.

List of abbreviations: CPE: carbapenemase-producing Enterobacteriaceae, MDR: multidrug-resistant bacteria, BMD: broth microdilution, MIC: minimum inhibitory concentration, PCR: Polymerase chain reaction, E.coli: Escherichia coli, CSF: cerebrospinal fluid, AST: Antimicrobial susceptibility testing, CIM: Carbapenem Inactivation Method, DDDs: defined daily dosages,

Funding: None.

Conflict of interest: None.

Ethical approval: All study procedures were as per ethical guidelines of the 1975 Declaration of Helsinki and approved by the ethical committee of Faculty of Medicine, Ain Shams University. (Ethical approval number: FWA 000017585).

HIGHLIGHTS

- The current study evaluated the diagnostic performance of rapid polymyxin NP test for the detection of colistin and polymyxin-resistance among 42 MDR-Enterobacteriaceae isolates and determined the frequency of mcr-1 and mcr-2 genes among them using conventional PCR.
- The rapid polymyxin NP test exhibited high specificity and sensitivity for diagnosing polymyxin resistance. 42.9% of the isolates were resistant to colistin, and 35.7% were resistant to polymyxin. The mcr-1 gene was more prevalent (40.5%) than the mcr-2 gene (11.9%).
- In conclusion, the rapid polymyxin NP test proved to be an easy and quick technique that can reliably detect both polymyxin-resistant and susceptible Enterobacteriaceae isolates. This test can be used for diagnostic purposes in laboratories with limited resources or infection control.

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