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Colistin Resistance among *Enterobacterales* Isolates: Underlying Mechanisms and Alternative Treatment Options

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Abstract

Global dissemination of multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative bacteria (GNB) such as carbapenemase-producing *Enterobacterales* has resulted in reviving colistin as a final therapeutic alternative. Colistin resistance foretold a catastrophe. We aimed to detect the rates of carbapenems and colistin resistance among hospital-acquired *Enterobacterales* species, verify the underlying mechanisms and provide antibiogram for colistin-resistant isolates. The collected *Enterobacterales* isolates were tested for their antimicrobial susceptibility by the disk diffusion method and agar dilution was utilized for both imipenem and colistin. The production of ESβLs and carbapenemases was phenotypically assessed by the combined disk (CDT) and modified carbapenem inactivation (mCIM) tests, respectively. Possible attributes for colistin resistance were explored by detection of both plasmid- and efflux pump-mediated mechanisms. By multiplex PCR assay, carbapenem resistance (*blaNDM-1* & *blaOXA-48*) and mobilized colistin-resistant-1 (*mcr-1*) genes were identified. A total of 160 *Enterobacterales* isolates were obtained of which 68.8% were MDR, 25% were XDR and 6.3% were pandrug-resistant (PDR) isolates with no statistically significant difference among *Enterobacterales* species (P> 0.05). Carbapenems resistance was detected in 41.3% (66/160) while colistin resistance was detected in 22% (36/160) of isolates. *Proteus mirabilis* expressed the highest rate of colistin resistance (100%; 16/16), followed by *Enterobacter aerogenes* (23.1%; 6/26), *E. coli* (13%; 6/46) and *K.pneumoniae* (11.1%; 8/72). One hundred percent (36/36) of colistin-resistant isolates proved efflux pump activity for colistin. However; only 2% (2/100) of tested *Enterobacterales* carried *mcr-1* gene through molecular analysis. Colistin-resistant isolates exhibited variable susceptibility to the tested antimicrobial agents of which fosfomycin was the highest (94.1%). Efflux pump activity played a major role for colistin resistance among *Enterobacterales* species and fosfomycin could be a promising therapeutic option.

Keywords: *Enterobacterales*, Carbapenems, Colistin, Efflux Pump & *mcr-1* Gene

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Abbreviations: MDR: Multidrug-resistant; XDR: Extensively- drug resistant; PDR: Pandrug- resistant; CCCP: Carbonyl cyanide 3 chlorophenyl hydrazone; ESβLs: Extended-spectrum cephalosporins; EPIs: Efflux pumps inhibitors; LPS: Lipopolysaccharide; mCIM: Modified carbapenem inactivation method; MIC: Minimal inhibitory concentration; GNB: Gram-negative bacteria.

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INTRODUCTION

Antibiotic resistance is a phenomenon closely associated with both antibiotics overuse and bacterial evolution that provide worrisome prospects for all of humanity. Emergence and dissemination of MDR and XDR organisms are superbugs associated with hospital-acquired infections (HAIs) resulting in elevated mortality, therapeutic complications and economic burdens. Annually, about 1.7 million patients acquire HAIs of which 6% die.[1]

In the 1990s, *Enterobacterales* started to develop a wide range of resistance to extended-spectrum cephalosporins (ESβLs), since then carbapenems have to be utilized more as a last resort to combat MDR GNB.[2] Overtime, the emergence of carbapenem resistance among *Enterobacterales* has become a serious health concern for both public healthcare authorities and practitioners.[3] Carbapenemases production, structural mutations of outer porins and efflux pumps are key mechanisms contributing to carbapenem resistance among *Enterobacterales*. Of these, carbapenemase production is the commonest.[4]

Carbapenemases are β-lactamase enzymes that can be categorized based on need to divalent cations for their activation into metallo-carbapenemases, MβLs, (zinc-dependent class B) and non-metallo-carbapenemases (zinc-independent classes A and D) that efficiently hydrolyze all beta-lactams. Two of most prevalent carbapenemases are bla_{NDM} (class B) and bla_{OXA-48} (Class D). Carbapenemases are mostly plasmid-mediated, which allows easier horizontal transfer and consequently leads to rapid spread of carbapenem resistance worldwide.[4]

According to the World Health Organization's (WHO) pathogen priority list, carbapenem-resistant *Enterobacterales* (CRE) are classified as "critical" antibiotic-resistant pathogens that pose a tremendous threat to public health.[5] Around 80% of GNB in humans belong to the *Enterobacterales* and are responsible for various illnesses as respiratory tract infections, urinary tract infections, bloodstream-associated infections (BSAIs), meningitis, sepsis, and other types of infections.[6]

Currently, serious challenges were raised and medical community can "beam back" to the pre-antimicrobial era owing to lack of effective therapeutic alternatives and limitations in novel antibiotic development.[7] Due to the concerning global rise in MDR and XDR *Enterobacterales*, healthcare practitioners have been forced to reintroduce colistin as a final resort option to combat potentially life-threatening infections.[6]

Colistin (polymyxin E) is a type of bactericidal peptide with polycationic properties that exhibit strong antimicrobial activity against *Enterobacterales*. As it binds to lipid A component of lipopolysaccharide (LPS), consequently, Ca++ and Mg++ that form bridges between LPS are displaced leading to disruption of bacterial membrane.[8] Likewise, as colistin use has increased, there has been a progressive increase in the prevalence of colistin resistance in the last few years, and the identification of underlying mechanisms is imperative.[8]

The intrinsic resistance to colistin among *Enterobacterales* was thought to be caused by chromosomal mutations in certain genes such as *pmrA/B, phoP/Q* and *mgrB* that encode regulatory proteins which govern transcription of enzymes involved in modifying the structure of LPS and thus, reducing the outer membrane's negative charge along with weakening the electrostatic attraction of polymyxins, thereby allowing the bacteria to resist the antimicrobial effects of colistin.[9]

The mobilized colistin resistance gene (*mcr-1*) which is plasmid-encoded was first reported in *E. coli* isolates obtained from livestock in China. Additionally, the same gene was detected in clinical isolates of *K. pneumoniae*, drawing attention to the emerged colistin-resistant bacteria.[10] Subsequent research on the genetic mechanisms underlying colistin resistance revealed nine other mcr genes, ranging from *mcr-2* to *mcr-10*, in different bacterial species. Despite this, *mcr-1* remains the most common globally detected gene.[11] The *mcr-1* gene encodes a zinc-dependant metalloenzyme which facilitates phosphoethanolamine transfer onto bacterial lipid A, thus leads to a lower binding affinity of colistin to its target site.[10]

Also, several studies have reported that efflux pumps are capable of reducing the susceptibility of bacteria to colistin.[12] Efflux pump inhibitors (EPIs), such as carbonyl cyanide 3 chlorophenyl hydrazone CCCP, 2, 4-dinitrophenol (DNP), omeprazole and verapamil, have been investigated as potential means of reversing colistin resistance.[13,14]

The aim of the study was to determine the prevalence of ESβLs and carbapenems resistance among clinical *Enterobacterales* isolated from HAIs at Menoufia University Hospitals (MUHs), investigate the rates of colistin resistance and explore the role of both plasmid and efflux pump-mediated mechanisms. Our findings might be beneficial to elucidate the pattern of antibiotic susceptibility observed in colistin-resistant *Enterobacterales* isolates, thus to provide alternative treatment options for critically-ill patients, coinciding with antimicrobial stewardship of our healthcare facility.

MATERIALS AND METHODS

Ethical approval and study design

The current cross-sectional study was conducted between March 2021 to August 2022 at the Faculty of Medicine Menoufia University, Medical Microbiology and Immunology Department, after obtaining approval from the Local Research Ethical Committee of Faculty of Medicine, Menoufia University (IRB No 3/2021MICR22). Informed consents were obtained from the study participants before involvement in this study.

Collection of clinical samples & identification of bacteria

Various clinical specimens were obtained from 360 patients who were admitted to different departments and ICUs of MUHs with variable clinical types of HAIs that became evident at least 48 hours after admission such as respiratory tract infections, urinary tract infections, wound infection, burn infections and bacteremia. *Enterobacterales* isolates were identified through culture onto MacConkey's, blood, nutrient and CLED media. Subsequently, the collected isolates were subjected to standard microbiological methods, which involved morphological and biochemical identification of different species.[15] All the obtained species were subjected to:

Antimicrobial susceptibility test

The disk diffusion screening method (Kirby Bauer method) was applied against different antibiotic disks (Oxoid, England) as per CLSI, 2022 guidance.[16] For imipenem and colistin, minimal inhibitory concentration (MIC) was determined by agar dilution method.[16] The obtained *Enterobacterales* isolates were categorized as: MDR was defined as isolate non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories, while XDR was used to describe an isolate non-susceptible to ≥ 1 agent in all antimicrobial categories but still sensitive to ≤ 2 categories whereas, PDR refers to non-susceptibility to all antimicrobial agents.[17]

Phenotypic detection of ESβLs production

ESβLs production among the isolated *Enterobacterales* species was detected using ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg) and aztreonam (30µg) disks. For zone diameter less than or equal 22 mm, 27 mm, 25mm and 27 mm, respectively to at least one disk, the isolates were considered as potential ESβLs producers (CLSI, 2022). Then, ESβLs production was confirmed by ceftazidime/clavulanic acid combined disk test (CDT). An increase of ≥5mm in the zone diameter around ceftazidime/clavulanic acid than around ceftazidime alone was interpreted as positive ESβLs production.[16]

Detection of carbapenemase production by phenotypic method

To confirm carbapenemase production when the tested isolate was non-susceptible to at least one of carbapenems, the modified carbapenem inactivation method (mCIM) was applied. The procedure was performed and interpreted as per CLSI, 2022 directions. A positive result was defined as an inhibition zone diameter of 6-15 mm or the presence of pinpointed colonies within a 16-18 mm zone. Whereas, a negative result was indicated by a clear zone of inhibition that measured ≥19 mm around meropenem disk.[16]

Demonstration of efflux pump inhibition by MIC reduction assay using CCCP as efflux pump inhibitor (EPI)

Two sets of Mueller-Hinton (MH) agar (Oxoid, England) were prepared: the first with colistin only (Sigma Aldrich; code: C4461-100MG), the second with colistin and CCCP (Sigma Aldrich; code: C2759). To prepare the stock solution of CCCP, a concentration of 5mg/mL was used, and it was dissolved in Dimethyl sulfoxide (DMSO; Sigma, 48216) (Concentration of DMSO 0.2%). The concentration of CCCP in the MH agar plates was adjusted to 10 mg/L and was constantly kept whilst, that of the colistin was serially increased. A positive result indicating efflux pumps was defined as an eight-fold or greater decrease in colistin minimum inhibitory concentration (MIC) on the addition of CCCP. To calculate the mean fold change, the following formula was used: "[1/total sample size (n)] × Σ (MIC fold change × frequency of fold change)" where the 'frequency of fold change' is the number of times a particular MIC fold change was recorded for that species.[13]

Molecular characterization of carbapenem & colistin resistance

One hundred of the isolated *Enterobacterales* spp. (40 *K. pneumoniae*, 24 *E. coli*, 20 *Enterobacter aerogenes* & 16 *Proteus mirabilis*) were investigated to determine whether the target genes were present (bla_{NDM-1} and bla_{OXA-48} for carbapenemase production & *mcr-1* for colistin resistance) by multiplex PCR assay. DNA extraction and purification for gene analysis were done in accordance with the manufacturer's instructions by the Gene JET Kit from Thermo Scientific (Thermo Fisher Scientific, USA, K0512). Sequence of primers for detection of bla_{NDM-1}, bla_{OXA-48}, and *mcr-1* genes are shown in Table 1.[18-20]

Statistical analysis

The data collected in this study were tabulated and statistically analyzed using a Statistical Package of Social Science (SPSS) version 29 and Epi Info 2000 programs, the Chi-square test or Fisher's-exact test were used as tests for significance of qualitative data. While quantitative data were assessed using student T-test. The differences between groups were considered significantly different with p-values smaller than 0.05.

RESULTS

A total 160 non-duplicate, consecutive *Enterobacterales* isolates were obtained from the study participants (n= 360) with a mean age 45.8±22.6 years, of which males represented 49.2% (177/360) while females were 50.8% (183/360). Nearly, 62 (38.75%), 28 (17.5%), 25 (15.6%), 20 (12.5%), 18 (11.25%) and 7 (4.4%) isolates were recovered from urine samples, sputum, pus, surgical drains or wound swabs, bronchial aspirate, blood and burn swabs respectively. The most frequently isolated microorganisms were *K. pneumoniae* (72/160; 45%), followed by *E. coli* (46/160; 28.75%), *Enterobacter aerogenes* (26/160; 16.25%) and *Proteus mirabilis* (16/160; 10%). Among patients infected with *Enterobacterales* species (n=160): 70 (43.75%), 55 (34.4%) and 5 (3.1%) patients have received cephalosporins, carbapenems and colistin therapy, respectively.

Regarding antimicrobial resistance pattern, the resistance rates reached 97.5%, 91.3%, 88.8%, 87.5%, 80.0%, 78.8%, 77.5%, 75.0%, 75.0%, 72.5%, 72.5%, 69.4%, 68.8%, 68.1%, 66.3%, 61.9%, 60.0%, 45%, 43.8%, 42.5%, 41.3%, 39.4% and 33.8% for cefixime, cefotaxime, cefoperazone,

Table 1. Sequence of primers for target genes in the study

Genes	Primers sequence (5'-3')	Product size	Reference
blaNDM- 1	F:TTGGCGATCTGGTTTTCC		
	R:GGTTGATCTCCTGCTTGA	195	(18)
blaOxa-48	F: TTGGTGGCATCGATTATCGG		
	R:GAGCACTTCTTTTGTGATGGC	744	(19)
mcr- 1	F:ATGCCAGTTTCTTTCGCGTG		
	R:TCGGCAAATTGCGCTTTTGGC	502	(20)

Table 2. Comparison between phenotypic screening and confirmatory tests for ESβLs and carbapenemase production among the isolated *Enterobacterales* species

Phenotypic tests For ESβL detection	*K. pneumoniae* (72)		*E. coli* (46)		*E. aerogenes* (26)		*P. mirabilis* (16)		Total *Enterobacterales*		P value **
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
Disk diffusion screening	70 (97.2%)	2 (2.8%)	44 (95.7%)	2 (4.3%)	26 (100%)	0 (0.0%)	16 (100%)	0 (0.0%)	156 (97.5%)	4 (2.5%)	.625
CDT	54 (75%)	18 (25%)	29 (63%)	17 (37%)	16 (61.5%)	10 (38.5%)	10 (62.5%)	6 (37.5%)	109 (68.1%)	51 (31.9%)	.413
P value *	0.0002		0.0002		0.0007		0.018		<.0001		
Phenotypic tests for carbapenem resistance	*K. pneumoniae* (72)		*E. coli* (46)		*E. aerogenes* (26)		*P. mirabilis* (16)		Total *Enterobacterales* (160)		
	R	S	R	S	R	S	R	S	R	S	
Disk diffusion	36 (50%)	36 (50%)	22 (47.8%)	24 (52.2%)	20 (76.9%)	6 (23.1%)	12 (75%)	4 (25%)	90 (56.2%)	70 (43.8%)	.055
Agar dilution (Imipenem MIC)	28 (38.9%)	44 (61.1%)	17 (37%)	29 (63%)	15 (57.7%)	11 (42.3%)	6 (37.5%)	10 (62.5%)	66 (41.3%)	94 (58.7%)	.32
P value ***	.179		.291		.139		.033		.007		
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
mCIM	26 (36.1%)	46 (63.9%)	17 (37%)	29 (63%)	15 (57.7%)	11 (42.3%)	6 (37.5%)	10 (62.5%)	64 (40%)	96 (60%)	.225

* Comparison between disk diffusion screening method and CDT by Fisher's exact test

** Comparison between different Enterobacterales species for each test.

*** Comparison between disk diffusion and agar dilution as screening methods for carbapenem resistance by Chi-square test.

ceftazidime, piperacillin, cefepime, azithromycin, ampicillin/sulbactam, ciprofloxacin, levofloxacin, gentamycin, norfloxacin, co-trimoxazole, aztreonam, piperacillin/tazobactam, amikacin, cefoxitin, doxycycline, ertapenem, meropenem, doripenem, imipenem and tigecycline. Isolates recovered from urine samples (n=62) were resistant to nitrofurantoin (80.6%) and fosfomycin (12.9%) (Figure 1). Out of the isolated *Enterobacterales* spp., 68.8% (110/160), 25% (40/160) and 6.3% (10/160) were MDR, XDR and PDR respectively but with no statistically significant difference among *Enterobacterales* spp. (P- value >0.05).

About 97.5% (156/160) of *Enterobacterales* isolates were ESβLs producers by disk diffusion screening test. Meanwhile, the CDT revealed only 68.1% (109/160) of them as positive ESβLs producers with a significance statistical difference between the two tests (P-value < 0.05) (Table 2 & Figure 2).

Nearly, 56.2% (90/160) of *Enterobacterales* isolates were carbapenem resistant by disk diffusion method compared to only 41.3% (66/160) by imipenem agar dilution method with a significance statistical difference (P- value < 0.05). Confirmatory test for carbapenemase detection (mCIM), detected 40% (64/160) of *Enterobacterales* spp. as carbapenemase producers (Table 2 & Figure 3).

About 31.9% (51/160) of the isolated *Enterobacterales* spp. were ESβLs/ carbapenemases co-producers, a statistically significant association was noticed between ESβLs and carbapenemases production among the tested isolates (P- value=0.01) (Figure 4).

Colistin resistance among *Enterobacterales* isolates by agar dilution method reached 22.2% (36/160). *Proteus mirabilis* isolates exhibited the the highest degree of colistin resistance (100%; 16/16), followed by *Enterobacter aerogenes* (23.1%; 6/26), *E. coli* (13%; 6/46) and the least resistance was for *K.pneumoniae* (11.1%; 8/72). Approximately 72.2% (26/36), 16.7% (6/36), and 11.1% (4/36) of the isolates showed MIC values at 64µg/ml, 16µg/ml, and 8µg/ml for colistin, respectively (Table 3).

Notably, when using CCCP as efflux pump inhibitor, it could drop the mean fold of colistin MIC for *K. pneumoniae* from 50 to 0.125, from 29.3 to 0.125 for *E. coli*, from 48 to 0.104 for *Enterobacter aerogenes* and from 58 to 0.56 for *Proteus mirabilis* (Table 3).

As regards distribution of carbapenemase genes among 100 *Enterobacterales* isolates, 35%

Table 3. Colistin MIC reduction assay when using CCCP as efflux pump inhibitor among colistin-resistant isolates (n=36)

Isolates (n=36)	MIC of colistin (µg/ml)	MIC of colistin + CCCP (Fold change)	Conclusion effect	Mean fold change of colistin MIC ± SD	Mean fold change of colistin + CCCP MIC	P value
***Klebsiella pneumoniae* (n=8)**						
6 isolates	64	0.125 (512)	Reverse	50 ± 25.9	0.125 ± 0	.00096
2 isolates	8	0.125 (64)	Reverse			
E. coli (n=6)						
2 isolates	8	0.125 (64)	Reverse	29.3 ±27.09	0.125 ± 0	.04592
2 isolates	16	0.125 (128)	Reverse			
2 isolates	64	0.125 (512)	Reverse			
***Enterobacter aerogenes* (n=6)**						
4 isolates	64	0.125 (512)	Reverse	48 ± 24.78	0.104± .032	<.001
2 isolates	16	0.125 (128)	Reverse			
***Proteus mirabilis* (n=16)**						
2 isolates	16	0.125 (128)	Reverse	58 ± 16.4	0.56 ± .84	< .00001
4 isolates	64	2 (32)	Reverse			
2 isolates	64	0.0625 (1024)	Reverse			
8 isolates	64	0.125 (512)	Reverse			

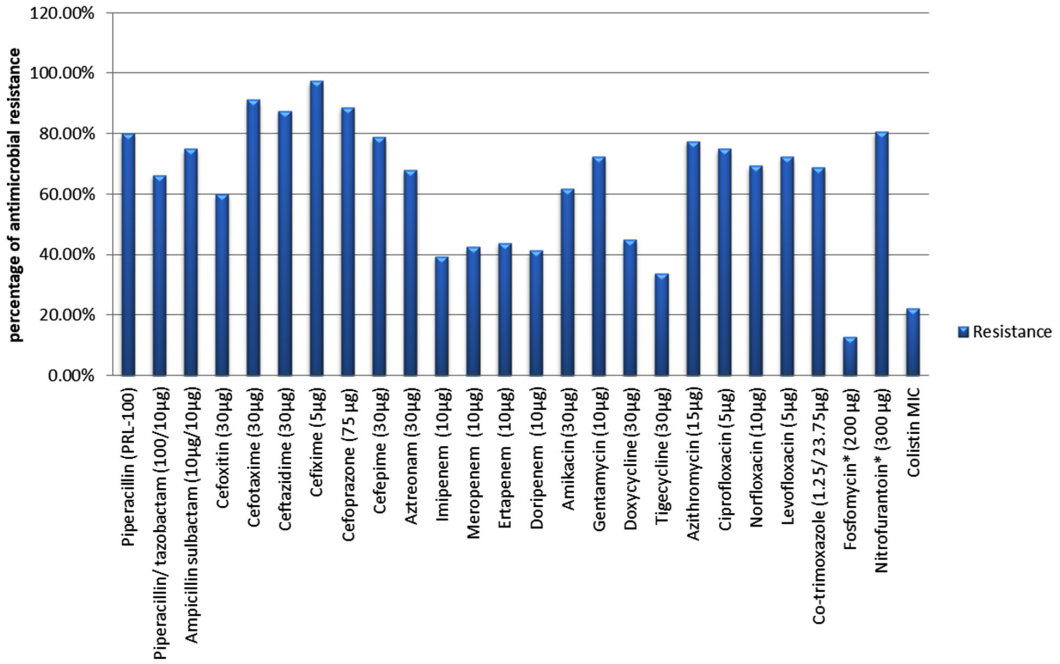


Figure 1. Antimicrobial resistance pattern of *Enterobacterales* isolates
*Fosfomycin and nitrofurantoin were tested against urine samples according to guidance of CLSI 2022

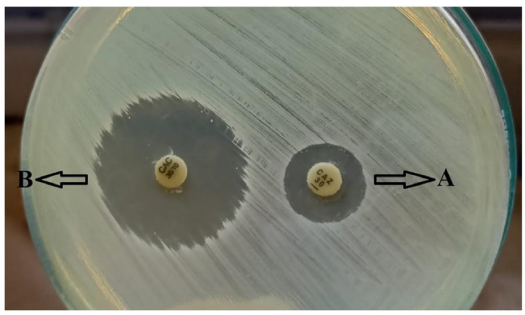


Figure 2. Combined disk test (CDT) for ESβLs production. Letter A represents ceftazidime alone. Letter B represents ceftazidime/clavulanate. There was an increase of inhibitory zone diameter ≥5 mm around ceftazidime/clavulanate (CAC) than ceftazidime alone.

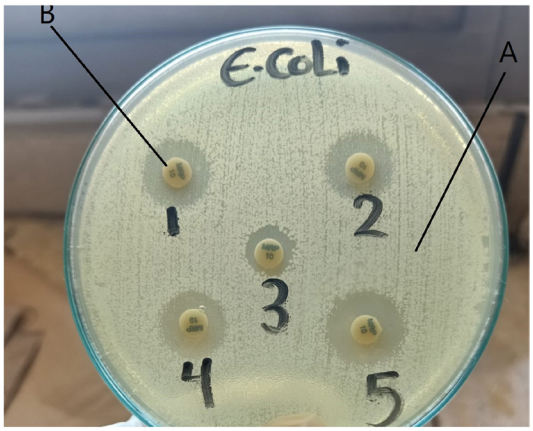


Figure 3. Detection of carbapenemase production by modified carbapenem inactivation test. Letter A represents the reference strain *E. coli* ATCC 25922 which is meropenem sensitive while letter B represents meropenem disk (10µg). Isolate 3 showed zone of inhibition less than 15mm and was considered as positive carbapenemase producer. Isolates 5 showed a zone of inhibition ≥19 mm that indicated a negative result.

(35/100) and 20% (20/100) were positive for bla_{NDM-1} and bla_{OXA-48}, respectively. Additionally, 16% (16/100) of them revealed co-existence of the two genes. The highest prevalence of bla_{NDM-1} was detected among *E. coli* (45.8%; 11/24) followed by *K. pneumoniae* (35%; 14/40), *Enterobacter aerogenes* (30%; 6/20) and finally *Proteus mirabilis* isolates (25%; 4/16) (P> 0.05). *E. coli* and *K. pneumoniae* showed equal frequency for bla_{OXA-48} gene (25% for each) followed by *Enterobacter aerogenes* (20%; 4/20) without significant difference as well (P> 0.05). As a genetic determinant of colistin resistance, *mcr-1* gene was only identified in 2% (2/100) of the tested isolates (only in *E .coli*) and none of the remaining species (98/100; 98%) harbored the gene (Figure 5 & Table 4).

Among colistin-resistant (n=36) *Enterobacterales*, 11/36 (30.6%) and 6/36 (16.7%) isolates were respectively positive for bla_{NDM-1} and bla_{OXA-48}. Moreover, 5/36 (13.9%) isolates displayed co-existence of the two carbapenemase genes (Figure 6).

The characteristics of colistin-resistant isolates, both phenotypic and genotypic, are detailed in table 5. Out of 36 colistin-resistant isolates, 17 isolates were recovered from urine, 6 from wound, 4 from burn, 4 from bronchial aspirates, 3 from blood and 2 from sputum specimens. The highest percentage of colistin-resistant isolates (47.2%; 17/36) was from ICUs. 77.8% (28/36) and 61.1% (22/36) were respectively ESβLs and carbapenemase producers. Furthermore, 33.3% (12/36) of colistin-resistant isolates were MDR, 38.9% (14/36) were XDR and 27.8% (10/36) displayed non susceptibility to all the tested antibiotics and were reported as PDR cases.

The antimicrobial susceptibility pattern of colistin-resistant *Enterobacterales* by disk diffusion is showed in table 6. The susceptibility rates reached 94.1% and 50%, respectively for fosfomycin and piperacillin/tazobactam, 38.9% for all of cefoxitin, aztreonam, imipenem and meropenem, 33.3% for each of ampicillin/sulbactam, amikacin, doripenem and norfloxacin. Much lower susceptibility rates were observed for piperacillin, cefepime, gentamycin, ertapenem and levofloxacin (27.8% for each). Only 22.2% of colistin-resistant isolates were susceptible to tigecycline and ciprofloxacin and about 16.7% were susceptible to each of co-trimoxazole and

Table 4. Distribution of *blaNDM-1, blaOXA-48* and *mcr-1* genes by multiplex PCR among *Enterobacterales* isolates

Target genes	*Enterobacterales* isolates (n=100)						
	K.pneumoniae (40)	*E. coli* (24)	*E.aerogenes* (20)	*P. mirabilis* (16)	Total (100)		
	+ve	+ve	+ve	+ve	+ve	-ve	
			Single gene				
blaNDM-1	6 (15%)	7 (29.2%)	2 (10%)	4 (25%)	19 (19%)	81 (81%)	
blaOXA-48	2 (5%)	2 (8.3%)	-	-	4 (4%)	96 (96%)	
mcr-1	0	2 (8.3%)	-	-	2 (2%)	98 (98%)	
			Co-existence				
blaNDM-1 & *blaOXA-48*	8 (20%)	4 (16.7%)	4 (20%)	-	16 (16%)	84 (84%)	
Total							P- value
blaNDM-1	14 (35%)	11 (45.8%)	6 (30%)	4 (25%)	35 (35%)	65 (65%)	.660
blaOXA-48	10 (25%)	6 (25%)	4 (20%)	-	20 (20%)	80 (80%)	.311
mcr-1	-	2 (8.3%)	-	-	2 (2%)	98 (98%)	.091

Table 5. Phenotypic and genotypic characteristics of colistin-resistant isolates (n=36)

Isolates (n=36)	Specimens	Departments	Colistin MIC (µg/ml)	Efflux pump activity	*mcr-1*	*blaNDM-1*	*blaOXA-48*	CDT	mCIM	Pheno-types
*K.pneumoniae*1	Urine	ICU	64	+	-	+	+	+	+	PDR
K.pneumoniae 2	Urine	ICU	64	+	-	-	+	+	+	XDR
K.pneumoniae 3	Wound	Surgery	64	+	-	-	-	+	+	XDR
K.pneumoniae 4	Burn swab	Burn unit	8	+	-	+	-	+	+	PDR
K.pneumoniae 5	Urine	Urology	8	+	-	+	+	+	+	XDR
K.pneumoniae 6	Urine	Paediatrics	64	+	-	+	+	+	+	PDR
K.pneumoniae 7	Bronchial aspirate	ICU	64	+	-	-	-	+	+	PDR
K.pneumoniae 8	Blood	ICU	64	+	-	-	-	+	+	XDR
E.coli 1	Urine	ICU	8	+	+	+	-	+	+	XDR
E.coli 2	Urine	ICU	16	+	+	-	-	+	+	XDR
E.coli 3	Urine	Internal medicine	64	+	-	-	-	+	-	PDR
E.coli 4	Burn swab	Burn unit	8	+	-	-	-	+	+	PDR
E.coli 5	Urine	Urology	64	+	-	-	-	+	+	PDR
E.coli 6	Urine	ICU	16	+	-	-	-	+	-	PDR
E.aerogenes 1	Sputum	Chest	64	+	-	+	+	+	+	PDR
E.aerogenes 2	Bronchial aspirate	ICU	16	+	-	-	-	-	-	MDR
E.aerogenes 3	Wound	ICU	16	+	-	-	-	+	+	XDR
E.aerogenes 4	Bronchial aspirate	ICU	64	+	-	-	-	+	-	PDR
E.aerogenes 5	Bronchial aspirate	ICU	64	+	-	+	+	-	+	MDR
E.aerogenes 6	Sputum	Chest	64	+	-	-	-	+	+	XDR
P. mirabilis 1	Urine	Urology	16	+	-	-	-	+	-	XDR
P. mirabilis 2	Urine	Urology	64	+	-	-	-	-	-	MDR
P. mirabilis 3	Blood	Urology	64	+	-	-	-	-	-	MDR
P. mirabilis 4	Wound	Surgery	64	+	-	-	-	-	-	MDR
P. mirabilis 5	Wound	Urology	64	+	-	-	-	+	-	XDR
P. mirabilis 6	Burn swab	Burn unit	64	+	-	-	-	+	+	XDR
P. mirabilis 7	Urine	Paediatrics	64	+	-	+	-	+	+	MDR
P. mirabilis 8	Urine	ICU	64	+	-	+	-	+	+	MDR
P. mirabilis 9	Urine	ICU	16	+	-	-	-	+	-	XDR
P. mirabilis 10	Urine	ICU	64	+	-	-	-	-	-	MDR
P. mirabilis 11	Blood	Paediatrics	64	+	-	-	-	-	-	MDR
P. mirabilis 12	Wound	Surgery	64	+	-	-	-	-	-	MDR
P. mirabilis 13	Wound	ICU	64	+	-	-	-	+	-	XDR
P. mirabilis 14	Burn swab	Burn unit	64	+	-	+	-	+	+	XDR
P. mirabilis 15	Urine	ICU	64	+	-	+	-	+	+	MDR
P. mirabilis 16	Urine	ICU	64	+	-	-	-	+	+	MDR
				36 (100%)	2 (5.6%)	11 (30.6%)	6 (16.7%)	28 (77.8%)	22 (61.1%)	
Total	Total MDR=12 (33.3%) Total XDR= 14 (38.9%) Total PDR=10 (27.8%)									

Table 6. Susceptibility profile of colistin-resistant *Enterobacterales* isolates (n=36)

Antimicrobial agents	Colistin-resistant *Enterobacterales* (n=36)		X^2	P value
	S (%)	R (%)		
Fosfomycin (200µg)*	16 (94.1%)	1 (5.9%)	0.26	
Piperacillin/ tazobactam (100/10µg)	18 (50.0%)	18 (50.0%)	6.517	.011
Cefoxitin (30µg)	14 (38.9%)	22 (61.1%)	0.0004	.984
Aztreonam (30µg)	14 (38.9%)	22 (61.1%)	3.871	.049
Imipenem (10µg)	14 (38.9%)	22 (61.1%)	4.815	.028
Meropenem (10µg)	14 (38.9%)	22 (61.1%)	2.783	.095
Ampicillin/ sulbactam (10µg/10µg)	12 (33.3%)	24 (66.7%)	5.308	.011
Amikacin (30µg)	12 (33.3%)	24 (66.7%)	0.084	.772
Norfloxacin (10µg)	12 (33.3%)	24 (66.7%)	2.376	.123
Doripenem (10µg)	12 (33.3%)	24 (66.7%)	5.887	.015
Levofloxacin (5µg)	10 (27.8%)	26 (72.2%)	.733	.392
Cefepime (30µg)	10 (27.8%)	26 (72.2%)	.733	.392
Piperacillin (PRL-100)	10 (27.8%)	26 (72.2%)	8.193	.004
Ertapenem (10µg)	10 (27.8%)	26 (72.2%)	8.065	.005
Gentamycin (10µg)	10 (27.8%)	26 (72.2%)	0.048	.827
Ciprofloxacin (5µg)	8 (22.2%)	28 (77.8%)	0.357	.55
Tigecycline (30µg)	8 (22.2%)	28 (77.8%)	32.385	< 0.001
Ceftazidime (30µg)	6 (16.7%)	30 (83.3%)	.755	.385
Cotrimoxazole (1.25/23.75 µg)	6 (16.7%)	30 (83.3%)	1.611	.204
Doxycycline (30µg)	4 (11.1%)	32 (88.9%)	< 0.00001	
Nitrofurantoin (300 µg)*	2 (11.8%)	15 (88.2%)	0.712	
Azithromycin (15µg)	2 (5.6%)	34 (94.4%)	0.25	
Cefotaxime (30µg)	0 0.0%	36 (100%)	1	
Cefixime (5µg)	0 0.0%	36 (100%)	1	
Cefoperazone (75 µg)	0 0.0%	36 (100%)	1	

*Among colistin-resistant isolates (n=36), 17 isolates were recovered urine samples and tested against fosfomycin and nitrofurantoin according to guidance of CLSI 2022

ceftazidime. The lowest susceptibility was for doxycycline, nitrofurantoin (11.1% for each) and azithromycin (5.6%). Notably, all (100%) isolates were resistant to cefotaxime, cefixime and cefoperazone.

DISCUSSION

Indiscriminate use of antibiotics resulted in worldwide dissemination of drug-resistant organisms of which carbapenem-resistant *Enterobacterales* are of major concern. This limits

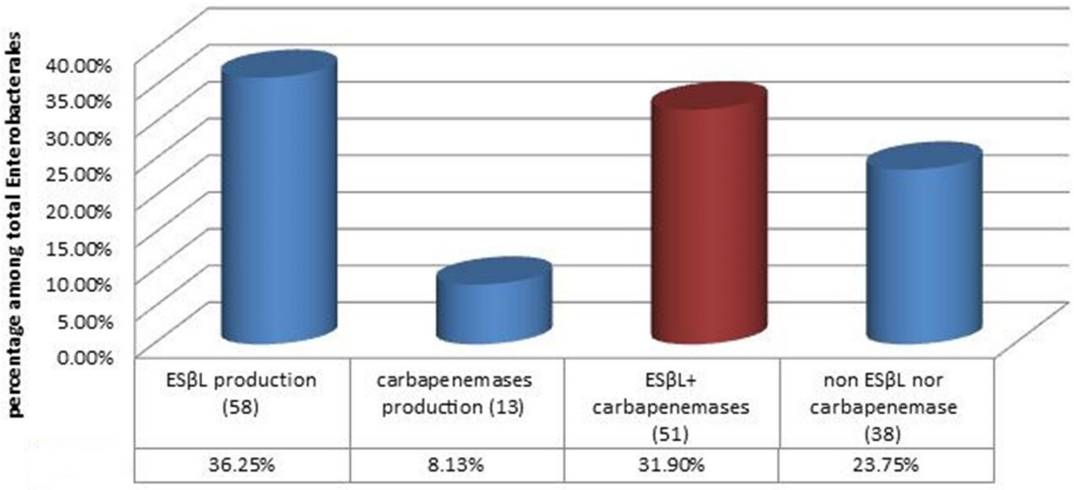


Figure 4. Association between ESβLs production (by CDT) and carbapenemases production (by mCIM) among *Enterobacterales* isolates (X2 =6.567 and P- value =.0104)

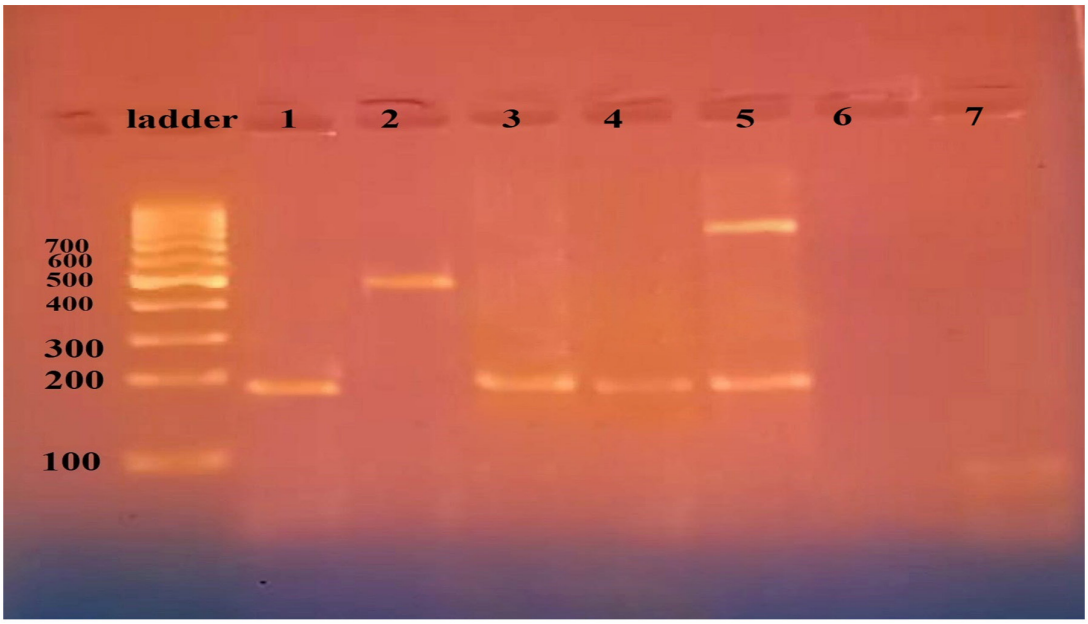
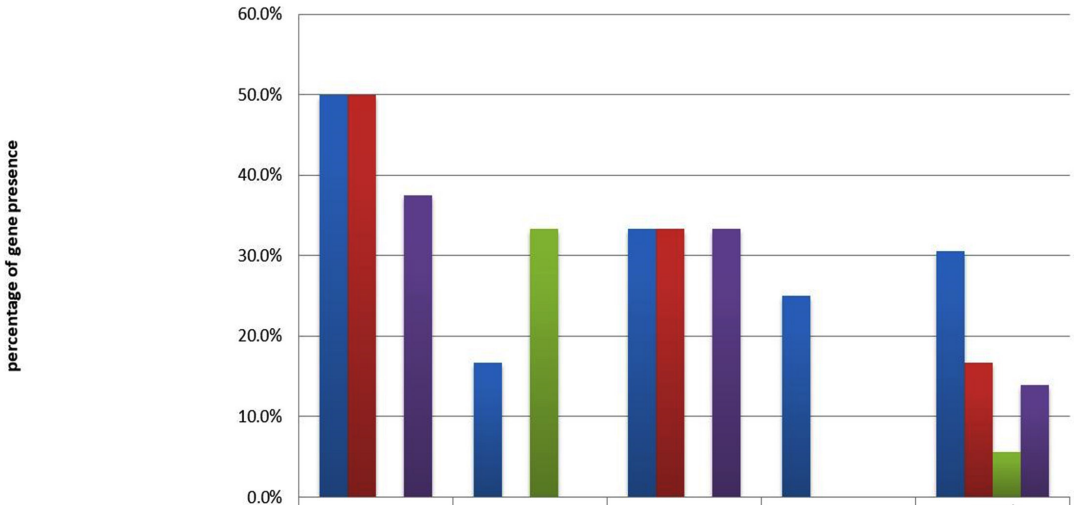


Figure 5. Agarose gel electrophoresis for the multiplex PCR-amplified products of *Enterobacterales* isolates. Ladder is DNA molecular size marker (100 bp). Lanes 1, 3 and 4, were positive for *blaNDM-1* (195bp), Lane 2 was positive to *mcr-1* (502), lane 5 was positive for *blaNDM-1* & *blaOXA-48* (744). Lanes 6 & 7 were negative



	K. pneumoniae (8)	*E. coli (6)*	*Enterobacter aerogenes (6)*	*P. mirabilis (16)*	*Total Enterobacterales (36)*
blaNDM-1	*50.0%*	*16.7%*	*33.3%*	*25.0%*	*30.6%*
blaOXA-48	*50.0%*	*0.0%*	*33.3%*	*0.0%*	*16.7%*
mcr-1	*0.0%*	*33.3%*	*0.0%*	*0.0%*	*5.6%*
Co-existence (blaNDM-1, blaOXA-48)	*37.5%*	*0.0%*	*33.3%*	*0.0%*	*13.9%*

Figure 6. Genetic profile for *blaNDM-1, blaOXA-48* and *mcr-1* among colistin-resistant *Enterobacterales* species

physicians' therapeutic options, particularly in developing nations where infectious diseases are abound and microbes exist in their most resistant phenotypes.[21]

The lack of new antibiotics led to reintroduction of old antimicrobials to treat severe infections. In that regard, colistin has gained clinical value owing to its activity against MDR GNB. However, there has been a significant increase in the frequency of colistin resistance in recent years.[22] Hence, we conducted this study to survey carbapenems and colistin resistance rates and the related mechanisms among *Enterobacterales* species isolated from patients with HAIs at MUHs, Egypt and assessed the antimicrobial susceptibility patterns of the recovered colistin-resistant isolates to provide alternative treatment lines.

In this study, 160 *Enterobacterales* isolates were tested against different antimicrobial agents. Even, among members of the same antibiotic class, different levels of susceptibility were detected. Collectively, 68.8%, 25%, and 6.3% of the tested species were respectively MDR, XDR, and PDR isolates. This came in agreement with a previous study that reported 65.7% of hospital-acquired *Enterobacterales* as MDROs.[23] A study conducted in Saudi Arabia revealed 57.3% and 3.5% of *Enterobacterales* clinical isolates as MDR and XDR organisms, respectively, but no PDRs were detected.[24] A higher prevalence of MDR hospital isolates (81.0%) was reported by other study.[25] Literature review summarized MDR rates among *Enterobacterales* in Egypt, from 30% to 70%.[26] The elevated rates of MDR infections observed in this study is ultimately attributed to the critically-ill status for most of the studied patients, empirical use of broad-spectrum antibiotics and poor hygienic conditions in developing countries.[26]

ESβLs production poses a great challenge in the management of *Enterobacterales* infections and is one of the major mechanisms for emergence of MDROs. Accordingly, the magnitude of ESβLs reached 68.1% in our study and *K. pneumoniae* proved the highest frequency (54/72; 75%). Varying rates for ESβLs-producing *Enterobacterales* were reported in the Northeast of Iran (50.8 %),[27] and in Saudi Arabia (51.4%).[24] Lower reports were declared in Brazil (21.3%),[27] Mexico (30.7%),[28]

and Zimbabwe (14%).[29] On the contrary, much higher rates were observed in Ethiopia (70.9%),[29] Germany (83.6%),[30] Congo (92%),[31] and Cambodia (93.4%).[32] One potential reason for the high incidence of ESβLs could be the selective pressure resulting from the widespread use of beta-lactam antibiotics as the primary treatment option for bacterial infections caused by *Enterobacterales* in African countries.[29]

Emergence of CRE has evolved into a formidable threat to community and they keep escalating trends stably during the later years. By 2022, carbapenem resistance accounted for 61.1% in Egypt.[33] In USA, the prevalence of CRE colonization varied widely from 1%–30.4%.[34] In the current study, 66/160 (41.3%) isolates were carbapenem resistant and 64/160 (40%) were carbapenemases producers by the mCIM method. Carbapenem resistance rate was relatively higher than other studies that reported carbapenem resistance range from 20–30% among *Enterobacterales*.[35] Makharita *et al*. in Egypt recorded 36.1% of *Enterobacterales* as carbapenem resistant,[36] which was lower than that reported in Sindh province of Pakistan (59%).[37]

Class B (bla_{NDM-1}) and D (bla_{OXA-48}) carbapenemase genes were identified in 35% and 20%, respectively, of the tested *Enterobacterales* isolates. The metallo-beta-lactamase bla_{NDM-1} gene proved the highest prevalence among *E. coli* isolates (45.8%). Several studies have addressed bla_{NDM-1} as the predominant carbapenemase gene in CRE.[22,38] Ongoing higher rates were detected by Wang *et al*.[39] who found bla_{NDM-1} in 75% of *E. coli* isolates and Chaudhary *et al*.[40] recognized bla_{OXA-48} in 32.6% of MDR *Enterobacterales* species.

Our results revealed 31.9% of *Enterobacterales* species as ESβLs/carbapenemase co-producers. Tayh *et al*.[41] reported 20% resistance to imipenem among ESβL-producing *Enterobacterales* isolates and Qadi *et al*.[1] found that 43.9% and 68.3% of ESβLs- producing *Enterobacterales* were respectively non-susceptible to imipenem and meropenem. The co-expression of ESβLs and carbapenemases β-lactamases has exacerbated the emergence of XDR clinical strains, which are challenging to manage and pose a significant threat due to the potential for clonal spread of these genes. It is critical to establish guidelines to prevent the misuse and overuse of antibiotics, particularly carbapenems beside infection control policies and vigilant surveillance on a routine basis.[41]

Colistin is a key drug for MDR GNB, so its resistance is a significant concern, owing to shortage of alternative therapies.[1] Our study found 36/160 (22.2%) *Enterobacterales* isolates were resistant to colistin. *Proteus mirabilis* exhibited the highest colistin resistance (16/16; 100%) which may be due to the modification of LPS of outer membrane.[42] The frequency of colistin resistance among other *Enterobacterales* species was 23.1% (6/26) for *Enterobacter aerogenes*, 13% (6/46) for *E. coli*, and 11.1% (8/72) for *K. pneumoniae*. In another publication by Mahmoud *et al*.[43] in Egypt, colistin resistance appeared in 42.9% of *K. pneumoniae* and *E. coli* isolates. In Greek, a report by Meletis *et al*.[44] examined 718 clinical *Enterobacterales* isolates of which 57 (7.9%) isolates were colistin resistant. In Italy, colistin resistance reached 24.7% and was as high as in Enterobacter spp. (47%) and *K. pneumoniae* (43%).[45] In Gaza, 41% of *Enterobacterales* isolates were classified as colistin resistant and the Proteus group exhibited the highest resistance to colistin, with a rate of 63.2%, followed by Serratia (57.1%). In contrast, Klebsiella isolates had the lowest resistance rate, with only 31.6% exhibiting resistance to colistin.[1]

According to the current results, genotypic surveys for plasmid-encoded genes, has identified *mcr-1* in only two *E. coli* isolates (2%; 2/100) and none of other species expressed this gene. Such finding came in line with Ejaz *et al*. who detected only 2.6% of MDR GNB harbouring *mcr-1* gene.[46] Other studies done in Pakistan and Iran, reported *mcr-1* in 3% and 3.2% of clinical isolates, respectively.[47,48] About emergence of colistin resistance gene (*mcr-1*) among colistin-resistant *K. pneumoniae* in Jordan, Gharaibeh *et al*. declared that only 1.1% of the tested isolates had *mcr-1* gene.[49] In Egypt, Zaki *et al*.[50] detected *mcr-1* in two isolates (one *E. coli* strain & one *K.pneumoniae* strains). Also, Ibrahim *et al*. reported *mcr-1* gene in 7.1% of *K.pneumoniae* isolates recovered from urinary tract infection of ICU-admitted 70 years male patient.[51]

Mobilized colistin resistance *mcr-1* gene revealed higher frequencies in Greece and Italy (43% and 20.8%, respectively)[44,45] with higher predominance in *E. coli* compared to other species.[52,53] Nevertheless, *mcr-1* gene is a particularly concerning public health issue, because it can be transmitted more easily across diverse bacteria by horizontal gene transfer than chromosomal colistin resistance genes.[54] Among *Enterobacterales E. coli* tops the list in rapid acquisition/transfer of resistance traits by horizontal gene transfer.[55] The *mcr-1* has likely been emerged and accelerated by the use of colistin on farms in China and Southeast Asia,[10] and subsequently spread to other countries.[52]

Efflux pumps allow bacteria to move antimicrobials agents out of cells leading to antimicrobial resistance. Efflux pump inhibitors (EPIs) inhibit efflux and could reverse antimicrobial resistance.[12] Our results demonstrated that 100% (36/36) of colistin-resistant isolates proved efflux pump activity against colistin when using CCCP as EPI. The Role of CCCP as EPI to rescue colistin susceptibility was studied by Baron and Rolain.[13] who reported that, CCCP was found to be effective in reversing colistin resistance in all investigated strains, and demonstrated ability to restore colistin susceptibility. Ni *et al*.[56] suggested that, this effect may be attributed to renewing the negative charges of outer membrane. Park and Ko[57] proposed that enhanced colistin activity in these cells could be related to a decrease in ATP synthesis caused by CCCP action.

The characteristics of colistin-resistant isolates, both phenotypic and genotypic, were also analyzed and revealed that 30.6% (11/36) and 16.7% (6/36) of them harbored bla_{NDM-1} and bla_{OXA-48} respectively. Zafer *et al*. found 9/40 and 7/40 of colistin-resistant isolates were positive for bla_{OXA-48} and bla_{NDM-1} genes, respectively.[22] In Jordan,[50] 19% and 11.5% of colistin-resistant *Enterobacterales* isolates were positive for bla_{OXA-48} and bla_{NDM-1}, respectively. The occurrence of colistin resistance in conjunction with carbapenemase genes poses significant risks in the use of carbapenems and colistin to combat infections.[58]

Our colistin-resistant strains were most frequently isolated from urine specimens (17/36; 47.2%) and 47.2% of colistin-resistant strains were obtained from ICUs' samples. Zafer *et al*. found colistin-resistant Klebsiella and *E. coli* among cancer patients were highly recovered from blood specimens (60%),[22] this could be owing to the cancer patients' neutropenic state, which favours GNB bloodstream infection treated with colistin. Panigrahi *et al.* noticed that 31.4% of colistin-resistant GNB were isolated from respiratory samples followed by 25% from blood samples among ICU patients.[59] Sorour *et al*. detected that ICUs were the highest frequent site (66.7%) for isolation of colistin-resistant GNB compared to other hospital departments.[60]

Also, we assessed the susceptibility profile of colistin-resistant *Enterobacterales* to provide antimicrobial stewardship team with data required for implementation of antibiotic policy in our healthcare facility. Colistin-resistant isolates showed a considerable high susceptibility to fosfomycin (94.1%), piperacillin/ tazobactam (50%), aztreonam, imipenem, meropenem and cefoxitin (38.9% for each of them). On other hand, lower susceptibility was observed against other antimicrobial agents ranging from 33.3% to 5.6%. Surprisingly, absolute non susceptibility was detected for each of cefotaxime, cefixime and cefoperazone. These findings were remarkably similar to those of Gharaibeh *et al*., who documented significant resistance to ceftazidime, tobramycin, and imipenem and average susceptibility to fosfomycin among colistin-resistant *Enterobacterales*.[49]

Both colistin and fosfomycin are considered a salvage treatment for MDR and XDR CRE.[61] It is worth noting that fosfomycin kept activity against strains of *mcr-1* gene carrying colistin-resistant *Enterobacterales*. As shown in our study, fosfomycin susceptibility among colistin-resistant *Enterobacterales* isolated from urinary tract infections was 94.1% (16/17). Other study showed that 83.3% of strains carrying *mcr-1* gene showed fosfomycin susceptibility.[62] Beyond UTI as the main focus of fosfomycin prescription, fosfomycin also showed excellent diffusion to various tissues. Thus, it should be considered for managing various other types of infectious caused by MDR, XDR *Enterobacterales*.[63]

CONCLUSION

Carbapenem and colistin resistance reached alarming rates in Egypt. The high prevalence of MDR and XDR among *Enterobacterales* isolates was concerning and their higher rate among colistin-resistant *Enterobacterales* adds another layer of concern to this escalating problem. Efflux pump is a major contributor to the emerged colistin-resistant *Enterobacterales*. Plasmid-borne colistin resistance is now spreading all over the world. The coexistence of class D bla_{OXA-48} and class B bla_{NDM-1} carbapenemases genes was notable among CRE isolates. Fosfomycin achieved excellent activity against colistin-resistant isolates. Immediate action to monitor the usage of antimicrobials, especially colistin, is a must.

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None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AFM, SAMA and ABM designed and contributed to all aspects of the study. AFL provided and analyzed clinical information of the studied participants. MEE and RME performed the experiments. AME interpreted the clinical and laboratory data, and wrote the manuscript. All authors reviewed, edited and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, Faculty of Medicine, Menoufia University, Egypt, with reference number 3/2021MICR22.

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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