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ORIGINAL ARTICLE

Prevalence of Integrons and *mcr*-mediated resistance among colistin-resistant *Enterobacterales* in hospitalized patients

[1]Dalia S. ElFeky[*], [2]Nahla A. Melake, [3]Abdulwahab Z. BinJomah, [4]Fatimah F. Al Hulaily, [4]Naif D. Almutairi, [5]Khawlah E. Aldilaijan, [6]Lubna S. Alsaleem

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Cairo, Egypt
[2]Department of Pathology, College of Medicine, Imam Mohammad Ibn Saud Islamic University, Riyadh, Saudi Arabia; Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Egypt
[3]Microbiology Department, Riyadh Regional Laboratory & Blood Bank, Riyadh, Saudi Arabia; College of Medicine, Alfaisal University, Riyadh, Saudi Arabia
[4]Microbiology Department, Riyadh Regional Laboratory & Blood Bank, Riyadh, Saudi Arabia
[5]Genetic Section, Research Department, Health Sciences Research Center, Princess Nourah bint Abdulrahman University
[6]Medical Student, College of Medicine, Imam Mohammad Ibn Saud Islamic University, Riyadh, Saudi Arabia

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****Corresponding Author:***
Dr. Dalia Saad ElFeky
Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Cairo, Egypt
Tel.: 0 115 008 4872
dalia_saadelfeky@kasralainy.edu.eg

ABSTRACT

Background: *Colistin is a last therapeutic option for carbapenem-resistant Enterobacterales (CRE) infections. Emergence of plasmid-mediated mcr colistin resistance genes poses a potential threat for treatment of these infections. Integrons are known for their central role in antibiotic resistance.* ***Objectives:*** *The aim of the study was to survey the prevalence of mcr-genes and integrons in colistin-resistant Enterobacterales isolates and to assess methods for colistin susceptibility testing.* ***Methodology:*** *Eighty-six colistin-resistant Enterobacterales strains isolated from different clinical samples of hospitalized patients at King Saud Medical City, Riyadh, from February 2020 to December 2021 were included in the study. Antimicrobial susceptibility testing (AST) was done using Phoenix BD, Sensititre broth microdilution test, and the standard broth microdilution method (BMD). Screening for mcr-1 and mcr-2 genes and class I, II and III integrons was done by PCR.* ***Results:*** *Majority of the colistin-resistant isolates (97.6%) were Klebsiella pneumoniae. Categorial agreement (CA) of Sensititre broth microdilution test and Phoenix BD test with the reference BMD method for colistin susceptibility was 95.3% and 89.5%, respectively. Only 3 isolates (3.5%) were found positive for mcr-1 gene. Class I and II integrons were detected in 79. % and 30.2% of the isolates, respectively.* ***Conclusion:*** *Sensititre broth microdilution test displayed a good performance for colistin AST, while Phoenix BD results should be confirmed by another method. Plasmid borne mcr-mediated resistance plays a minor role in colistin resistance among Klebsiella pneumoniae isolates. The alarming high prevalence of antibiotic resistant class I and II integrons in the studied isolates warrants further investigations.*

INTRODUCTION

For most carbapenem resistant Gram-negative bacilli (GNB), colistin (polymyxin E) is the last line antibiotic[1]. Among the five polymyxins (A–E), colistin (polymyxin E) and polymyxin B have been used in clinical settings. However, due to their nephrotoxicity and neurotoxicity actions, and availability of comparatively ‘safer’ drugs such as beta-lactams, they were no longer used[2]. In 1990s, polymyxins were reintroduced to counter the uncontrolled spread of carbapenem-resistant bacteria, despite their toxic effects being at a standstill[3].

Polymyxin-resistance is rising rapidly with increase in the clinical use of polymyxins. There have been reports of colistin-resistant and even pan-drug-resistant GNB[1]. Resistance to colistin is usually chromosomally mediated and not transmissible between bacteria. However, the discovery of *mcr-1* plasmid-mediated colistin resistance mechanism is of great importance for the longevity of colistin due to its potential for spreading among clinical pathogens [1]. In the last few years, more than 40 countries on five continents have reported variants of *mcr-1*(*mcr*-1-9)[4].

Integrons have been well studied and documented for their role in antibiotic resistance. An integron is a non-mobile genetic element that can be transferred

between bacteria *via* transposons and plasmids[5]**.** The essential components of the integron include an integrase gene, *intI,* an adjacent recombination site, *attI*, and a promoter region from which integrated gene cassettes are expressed. The gene cassettes are located within the variable region and are integrated in tandem at the *attI* site**.** According to the amino acid sequences of *IntI* integrases, integrons have been divided into up to now, more than 9 classes, but only 4 main classes are associated with clinical isolates[6]**.** The Most predominant integrons found in *Enterobacterales* as well as other clinically significant GNB are class I integrons. Still, there is no enough information available on spread of integrons classes and their association with multidrug resistance (MDR) in GNB[7]**.**

In the recent document proposed by the CLSI/EUCAST Polymyxin Breakpoints Working Group, broth microdilution method (BMD) is considered the optimal method for testing colistin susceptibility[8]. Few studies have been carried out to date evaluating the performance of different commercially available colistin susceptibility methods with opposing results. Therefore, more studies are needed to establish which method is most accurate.

The current study was conducted to assess 2 commercially available methods used for MIC determination of colistin, and to detect the presence of plasmid-mediated colistin resistance genes *mcr-1* and *mcr-2* as well as class I to III integrons among colistin-resistant *Enterobacterales* isolates.

METHODOLOGY

This study has been approved by Institutional Review Board (IRB) committee of Princess Nourah Bint Abdul Rahman University (PNU) (**IRB reference no. 19-0058**) as well as King Saud Medical City (KSMC), Riyadh, Saudi Arabia (**IRB reference no. H1RI-26-Jan20-01).** The procedures used in this study adhere to the tenets of the Declaration of Helsinki. Patients' demographic and clinical data were obtained from KSMC data base. The practical part of the study was conducted in the Health Sciences Research Centre (HSRC), PNU, Riyadh, Saudi Arabia.

Bacterial isolates identification and antimicrobial susceptibility testing:

Eighty-six non-duplicate colistin-resistant *Enterobacterales* strains isolated from different clinical samples of hospitalized patients in KSMC during the period from February 2020 to December 2021 were included in the study. KSMC is a large tertiary hospital that contains the Riyadh Regional Laboratory. Identification and antimicrobial susceptibility testing (AST) of the isolates were done by BD Phoenix (BD Diagnostics, USA) using BD Phoenix NMIC/ID-431 panel which includes colistin in a concentration range of 0.5-4 µg/mL. Colistin-resistance was defined by the CLSI guidelines (Intermediate ≤ 2µg/mL, Resistant ≥4µg/mL)[9]. Members of *Enterobacterales* with intrinsic-resistance to colistin (*Morganella morganii, Proteus* and *Providencia species* as well as *Serratia marcescens*) were excluded. Sensititre® GNX2F (Thermo scientific, UK) was used for AST of the isolates as well. It includes colistin in a concentration range 0.25-4 µg/mL and Polymyxin B. The standard BMD was used for colistin susceptibility testing, using colistin sulphate powder (ACROS Organics, China) according to CLSI guidelines[9]. *E. coli* ATCC 29533 was used as a control for AST. All isolates were stored in glycerol broth stocks at -80°C until further molecular testing.

Molecular testing:

i. **DNA extraction:** Thermal extraction of DNA from isolates was performed by picking 3-5 colonies from freshly prepared agar plates, emulsifying them into sterile molecular-grade water and then the suspension was incubated at 95 °C for 15 minutes. This was followed by centrifugation at 10000 rpm for 10 minutes. The supernatant was transferred to a new tube and used as DNA template[10]. An *mcr*-1 positive *E coli* isolate (SA186) characterized in a previous study[11] was used as a positive control for PCR.

ii. **Screening for *mcr-1* and *mcr-2* genes:** Multiplex PCR amplification of *mcr-1* and *mcr-2* genes was done using previously published primers shown in table (1)[12]. The reaction was carried out in a total volume of 25µl containing 12.5µl of HotStarTaq® Master Mix Kit (Qiagen, Germany), 1µl of each forward and reverse primers (10mM) of each gene, 1µl of DNA template and 7.5µl of nuclease-free water. The reaction was carried out under the following conditions: initial denaturation at 95°C for 15min, 25 cycles of denaturation at 94°C for 30s, annealing at 58°C for 90s and elongation at 72°C for 60s, and a final cycle of elongation at 72°C for 10min. A positive control (*E coli* isolate SA186) and a negative control were included in each run. The amplicons were undergone electrophoresis using 1.5% agarose gel containing 4µL/100mL ethidium bromide at 90 V followed by visualization and imaging in the BioRad gel documentation system (Gel Doc XR System).

iii. **Screening for Class I, II and III integrons and their gene cassettes:**
The presence of class I, II and III integrons was studied by multiplex PCR assay using previously published primers (intI1, intI2, and intI3) [7,13] specific for integrases genes of integrons (Table 1). For gene cassette characterization, variable regions of class I and II integrons were amplified by monoplex PCR in isolates tested positive for the corresponding integron using previously published primers[7,13] (Table 1). For both reactions, a total volume of 20µl

was prepared containing 4µL of 5x FIREPol® Master Mix Ready to Load (Solis BioDyne), 0.5µl of each forward and reverse primers (10mM) of each gene, 3µl of DNA template and nuclease-free water to complete the reaction volume. For the multiplex PCR, the reaction was carried out under the following conditions: initial denaturation at 94°C for 5min, 32 cycles of denaturation at 94°C for 1min, annealing at 60°C for 1min and elongation at 72°C for 2min, with a final cycle of elongation at 72°C for 10min. For amplification of the variable region, the reaction was carried out under the following conditions: initial denaturation at 94°C for 5min, 35 cycles of denaturation at 94°C for 1min, annealing at 58°C for 1min and elongation at 72°C for 2min, with a final cycle of elongation at 72°C for 10min. The amplicons were undergone electrophoresis using 1.5% agarose gel containing 4µL/100mL ethidium bromide at 90V followed by visualization and imaging in the BioRad gel documentation system (Gel Doc XR System).

Table 1: Primers used in the study:

Primers for detection of *mcr-1* and *mcr-2* genes[12]		
Primer name	**Sequence (5'-3')**	**Amplicon Size (bp)**
mcr1-F	AGTCCGTTTGTTCTTGTGGC	320
mcr1-R	AGATCCTTGGTCTCGGCTTG	
mcr2-F	CAAGTGTGTTGGTCGCAGTT	715
mcr2-R	TCTAGCCCGACAAGCATACC	
Primers for detection of integrons and gene cassettes[7, 13]		
Primer name	**Sequence (5'-3')**	**Size (bp)**
IntI1-F	GGT CAA GGA TCT GGA TTT CG	436
IntI1-R	ACATGCGTGTAAATCATCGTC	
IntI2-F	CAC GGA TAT GCG ACA AAA AGG	788
IntI2-R	TGTA GCA AAC GAG TGA CGA AAT G	
IntI3-F	AGT GGG TGG CGA ATG AGT G	600
IntI3-R	TGT TCT TGT ATC GGC AGG TG	
Variable 5'CS	GGC ATC CAA GCA GCA AG	Variable
3'CS	AAG CAG ACT TGA CCT GA	
Variable attI2-F	GAC GGC ATG CAC GAT TTG TA	Variable
orfX-R	GAT GCC ATC GCA AGT ACG AG	

Statistical Analysis:

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 28 (IBM Corp., Armonk, NY, USA). Data was summarized using mean, standard deviation, median, minimum, and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. For comparing categorical data, Chi square ($\chi 2$) test was performed. Exact test was used instead when the expected frequency is less than 5[14]. P-values less than 0.05 were considered as statistically significant. Standard diagnostic indices including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic efficacy were calculated as described by **Galen**[15].

Regarding colistin MIC testing, categorical agreement (CA) was measured between the standard method (BMD) and the other two methods (Sensititre broth microdilution and Phoenix BD). CA is calculated as the percentage of isolates with result in the same category as the reference method, taking all isolates tested as denominator. Essential agreement (EA) could not be calculated due the different range of MIC detected by the standard BMD (0.125-128 µg/mL) and both Sensititre test (0.25-4µg/mL) and Phoenix BD (0.5-4µg/mL). Very major error (VME: false-susceptible) and major error (ME: false-resistance) were calculated for Sensititre test and Phoenix BD. As per CLSI guidelines, any test with both CA and EA greater than 90% can be considered a reliable alternative to the reference test, while tests with VME and ME greater than 3 are not acceptable[16]. Regarding other shared antibiotics between Phoenix BD and Sensititre test, only the CA was used since VME and ME could not be calculated due to the lack of a reference AST method for these antibiotics in this study.

RESULTS

This study included 86 non-repetitive colistin-resistant *Enterobacterales* isolates detected by Phoenix

BD isolated from different clinical specimens of hospitalized patients at KSMC during the study period. *Klebsiella pneumoniae* (*K. pneumoniae)* represent 97.6% of the colistin-resistant CRE isolates, while the rest of the isolates were *K. oxytoca* (1.2%) and *Enterobacter cloacae* (1.2%). The age of studied patients (no. = 86) ranged from 1 to 88 years (mean 46.73 ± 20.21 years). Majority of the isolates were from patients in the age group from 40-59 years old (41.9%) followed by elderly patients over 60 years old (27.9%). Majority of the patients (60.5%) were males. Most of the isolates (n= 80; 93%) were collected from ICU (n=45; 52.3%) and medical departments (n=35; 40.7%). Only 7% of the isolates were from surgical department. Majority of the colistin-resistant isolates included in the study were isolated from respiratory specimens (n=36; 41.8%) including sputum (24.4%), and endotracheal aspirate/bronchial wash (17.4%) followed by blood (n=16; 18.6%), pus/wound specimens (n=14; 16.4%), urine (12.8%), rectal swab specimens (8.1%) and lastly tissue specimens (2.3%).

MICs of *E. coli* ATCC 25922 for the tested antibiotics by both Sensititre test and Phoenix BD were all within the expected range as per both manufacturers' instruction. Table (2) shows that all the studied isolates (no. = 86 isolates) show complete resistance to meropenem, ertapenem and ciprofloxacin by both methods. Figure (1) shows that all isolates show complete resistance to cefepime, piperacillin-tazobactam, and levofloxacin when tested by Sensititre test while the resistant rate was 98.8% when tested by Phoenix BD for piperacillin-tazobactam and levofloxacin and 96.5% for cefepime. Majority of the isolates were sensitive to tigecycline (84.9%) followed by amikacin (10.5%) as detected by Sensititre test, while for Phoenix BD, rate for tigecycline sensitivity was 53.5% followed by gentamycin (32.6%) and amikacin (25.6%). CA between Phoenix BD test and Sensititre test is ≥90% for most of the common antibiotics tested by both methods, however, CA is only 62% for tigecycline, 79% and 71% for amikacin and gentamycin, respectively, and 85% for trimethoprim-sulfamethoxazole (SXT).

Table 2: Categorical agreement between Sensititre broth microdilution test and Phoenix BD test for the common antibiotics

Antibiotic	AST Test	No. (%) of isolates			CA*
		Sensitive	Intermediate	Resistant	
Ceftazidime	**Sensititre broth microdilution**	1 (1.2)	2 (2.3)	83 (96.5)	92%
	Phoenix BD	2 (2.3)	4 (4.7)	80 (93)	(79/86)
Cefepime	**Sensititre broth microdilution**	0 (0.0)	0 (0.0)	86 (100)	96.5%
	Phoenix BD	1 (1.2)	2 (2.3)	83 (96.5)	(83/86)
Imipenem	**Sensititre broth microdilution**	0 (0.0)	1 (1.2)	85 (98.8)	98.8%
	Phoenix BD	0 (0.0)	0 (0.0)	86 (100)	(85/86)
Meropenem	**Sensititre broth microdilution**	0 (0.0)	0 (0.0)	86 (100)	100%
	Phoenix BD	0 (0.0)	0 (0.0)	86 (100)	(86/86)
Ertapenem	**Sensititre broth microdilution**	0 (0.0)	0 (0.0)	86 (100)	100%
	Phoenix BD	0 (0.0)	0 (0.0)	86 (100)	(86/86)
Aztreonam	**Sensititre broth microdilution**	1 (1.2)	0 (0.0)	85 (98.8)	97.7%
	Phoenix BD	3 (3.5)	0 (0.0)	83 (96.5)	(84/86)
Piperacillin-Tazobactam	**Sensititre broth microdilution**	0 (0.0)	0 (0.0)	86 (100)	98.8%
	Phoenix BD	1 (1.2)	0 (0.0)	85 (98.8)	(85/86)
Amikacin	**Sensititre broth microdilution**	9 (10.5)	0 (0.0)	77 (89.5)	79%
	Phoenix BD	22 (25.6)	1 (1.2)	63 (73.3)	(68/86)
Gentamycin	**Sensititre broth microdilution**	5 (5.8)	0 (0.0)	81 (94.2)	71%
	Phoenix BD	28 (32.6)	0 (0.0)	58 (67.4)	(61/86)
Ciprofloxacin	**Sensititre broth microdilution**	0 (0.0)	0 (0.0)	86 (100)	100%
	Phoenix BD	0 (0.0)	0 (0.0)	86 (100)	(86/86)
Levofloxacin	**Sensititre broth microdilution**	0 (0.0)	0 (0.0)	86 (100)	98.8%
	Phoenix BD	0 (0.0)	1 (1.2)	85 (98.8)	(85/86)
Trimethoprim-Sulfamethoxazole	**Sensititre broth microdilution**	5 (5.8)	0 (0.0)	81 (94.2)	85%
	Phoenix BD	14 (16.3)	0 (0.0)	72 (83.7)	(73/86)
Tigecycline	**Sensititre broth microdilution**	73 (84.9)	9 (10.4)	4 (4.7)	62.8%
	Phoenix BD	46 (53.5)	35 (40.7)	5 (5.8)	(54/86)

**CA: categorical agreement*

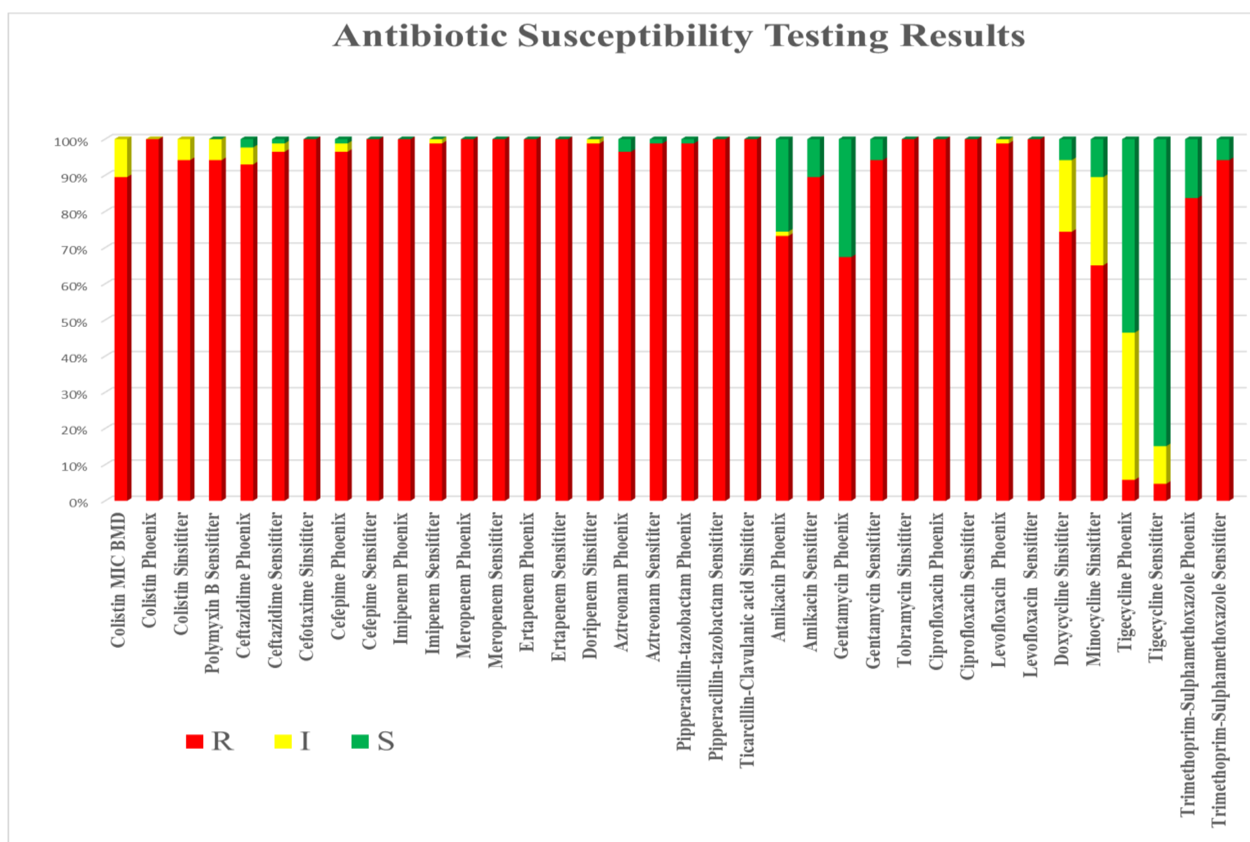

Fig. 1: Results of antibiotic susceptibility testing of the isolates

Colistin susceptibility testing of colistin-resistant isolates detected by Phoenix BD test included in this study was assessed additionally by BMD and Sensititre test. BMD was considered as the gold standard method as recommended by both CLSI and EUCAST[10]. MICs of *E. coli* ATCC 25922 for colistin was between 0.25 and 0.5 µg/mL by all testing methods, which is within the expected range (0.25-2µg/mL for colistin and polymyxin B). The MIC50 and MIC90 values for the tested isolates as measured by BMD were found to be 32µg/mL and 128µg/mL, respectively. Table (3) shows the results of the Phoenix BD test and Sesititre test in comparison with the gold standard method (BMD). It shows that among the 86 colistin-resistant isolates tested by Phoenix BD test, 9 isolates (10.5%) were shown to be intermediate (≤2ug/mL) by the BMD, and among them 5 (5.8%) were tested intermediate by Sensititre test as well. CA of Sensititre test with the reference BMD method was ≥90% (95.3%) while that of Phoenix BD test was 89.5%. ME rates for Sensititre test and Phoenix BD test were 4.6% and 10.5%, respectively which are both higher than the acceptable range according to the CLSI guidelines. There was no VME for Sensititre test, while it cannot be calculated for Phoenix BD test, since all the studied isolates are tested resistant by Phoenix BD.

Table (4) shows the accuracy indices of Phoenix BD test and Sensititer test when considering the standard BMD method as the gold standard method. It shows that both methods have 100% sensitivity; however, Sensititre has a higher PPV than Phoenix BD test (95.06% vs 89.53%). Regarding the specificity, Sensititre has 56% specificity and 100% NPV while the specificity and NPV could not be calculated for Phoenix BD since all the included isolates in the study were colistin-resistant by Phoenix BD test. Sensititre test has an accuracy of 95.35% compared to 89.53% for Phoenix BD test. Regarding polymyxin B susceptibility results as detected by Sensititre test, 5 isolates (5.8%) were tested intermediate, all of them were tested colistin intermediate by Sensititre test as well. The rest of the isolates were polymyxin-resistant.

Table (3) Categorical agreement, very major error, and major error rates of Sensititre broth microdilution and Phoenix BD methods in comparison with the reference BMD method for colistin MIC testing

AST Test	No. (%) of isolates		CA*	ME**	VME**
	Intermediate	Resistant			
BMD	9 (10.5 %)	77 (89.5%)			
Sensititre broth microdilution	5 (5.9%)	81 (94.2%)	95.3% (82/86)	4.6% (4/86)	0 %
Phoenix BD	0(0%)	86 (100%)	89.5% (77/ 86)	10.5 % (9/86)	-

** CA: categorical agreement*
***ME; major error*
****VME; very major error.*

Table (4) Accuracy indices of Phoenix BD and Sensititre broth microdilution for colistin MIC testing

Statistic	Phoenix BD test		Sensititre broth microdilution test	
	Value	95% CI	Value	95% CI
Sensitivity	100.00%	95.3- 100.00%	100.00%	95.32- 100.00%
Specificity		0.00 -33.6%	55.56%	21.20- 86.30%
Positive Predictive Value (PPV)	89.53%	89.53- 89.53%	95.06%	90.27- 97.56%
Negative Predictive Value (NPV)			100.00%	
Accuracy	89.53%	81.06- 95.10%	95.35%	88.52- 98.72%

Multiplex PCR for *mcr* genes (figure 2A) reveal that three isolates (3.5%) were tested positive for *mcr-1* gene, while none of the isolates harbors *mcr-2* gene. Regarding the *mcr-1* positive isolates, all of them are *K. pneumoniae* isolated from ICU patients. Two of the isolates were isolated from respiratory specimens (sputum and bronchial washings) of old patients aged 56-65 years, while the third one was isolated from the blood of a 15-year-old patient. Colistin MIC of the three isolates ranges from 8-16 µg/mL as detected by BMD. All the three isolates were sensitive to tigecycline, while two of them were sensitive or intermediate to both minocycline and doxycycline and one was sensitive to both amikacin and gentamycin as detected by Phoenix BD test.

Regarding screening for introns (figure 2B), integron I was amplified in a total of 68 isolates (79.1%) including 67 *K. pneumoniae* isolates and the only *E. cloacae* isolate, while integron II was amplified in a total of 26 isolates (30.2%), all of them were *K. pneumoniae* and contain integron I as well. It was noted that for integron II, a band of 2000bp was amplified in most of the positive samples (n=23), while the expected band of 788bp was amplified in 2 samples, and both bands were amplified in one sample. To confirm the specificity of the 2000 bp band for integron II, positive samples for that band were tested by monoplex PCR for each of the integron genes (I-III) separately, and the band was amplified only with Int I- 2 primers. Amplification of the variable region for integron I (figure 2C) reveal 1-4 bands (table 5A) with different combination of 7 different bands (200bp, 300bp, 600bp, 800bp, 1200bp, 2000bp, and 3000bp). The most common of them was a single band at 800 bp and 2 bands at 800+1200bp (10.3% for each). No band was obtained in one of the positive samples. On the other hand, amplification of the variable region for integron II (figure 2D & table 5B) reveals a single band at 1300bp in all the positive isolates except one isolate where 2200bp band was amplified, while no band was obtained in one of the positive samples. Since all the isolates included in the study were MDR with low frequency of sensitivity to most antibiotics, the association between integrons existence and antibiotic resistance among the tested isolates was calculated only for amikacin, gentamycin, minocycline, SXT and tigecycline. Significant association was found only between resistance to SXT and the presence of integron II (p-value=0.008).

Table 5: Characterization of gene cassettes of integron I and integron II positive samples

A. Variable regions of integron class I cassettes in integron 1 positive isolates		
No of bands	**Band size**	**No. of isolates (%)**
1	800	7 (10.3%)
1	1200	2 (2.9%)
1	2000	2 (2.9%)
2	800-1200	7 (10.3%)
2	300-800	6 (8.8%)
2	200-800	6 (8.8%)
2	1200-2000	5 (7.4%)
2	800-2000	2 (2.9%)
3	200-300-800	6 (8.8%)
3	200-600-800	4 (5.9%)
3	800-1200-2000	4 (5.9%)
3	200-800-1200	2 (2.9%)
3	200-800-2000	2 (2.9%)
4	200-300-800-1200	3 (4.4%)
4	200-300-800-3000	3 (4.4%)
4	200-800-1200-2000	3 (4.4%)
4	200-300-800-2000	2 (2.9%)
4	300-800-1200-2000	1 (1.5%)
No band		1 (1.5%)
Total of positive isolates		68 (100%)
B. Variable regions of integron class II cassettes in integron 2 positive isolates		
No of bands	**Band size**	**No. of isolates (%)**
1	1300	24 (92.3%)
1	2200	1 (3.9%)
No band		1(3.9%)
Total of positive isolates		26 (100%)

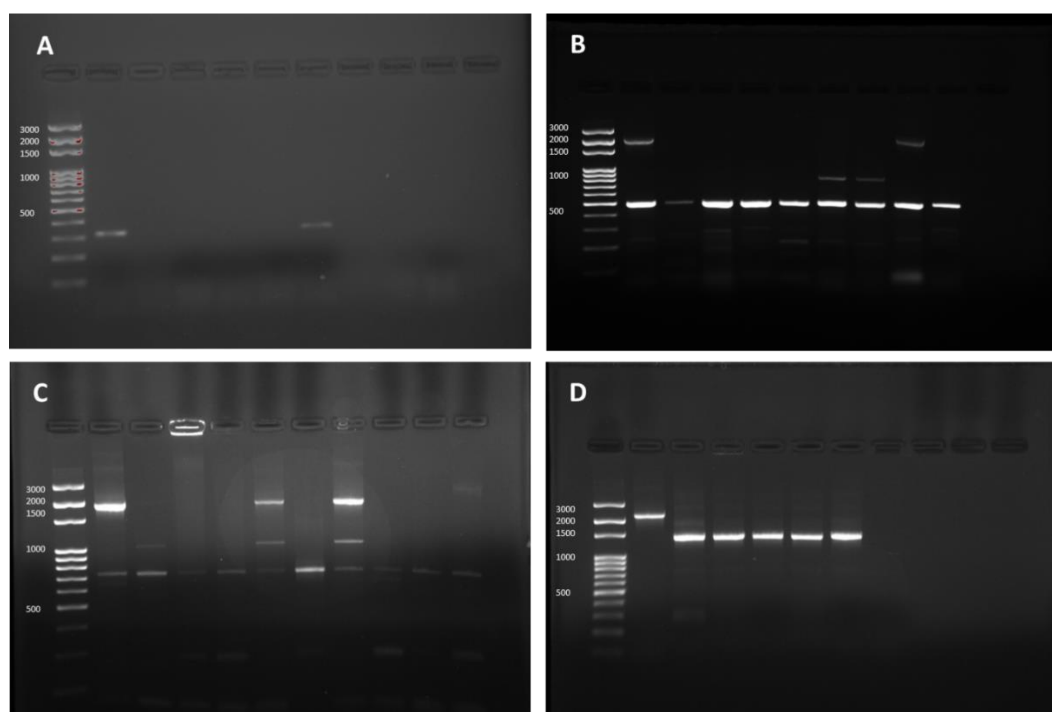

Figure (2): "A": Multiplex PCR for mcr-1 and mcr-2 genes. Lane 1: DNA ladder, Lane 2: mcr-1 positive control. Lane 7: Clinical isolate positive for mcr-1 gene. Lane 3-6 & 8-10: Clinical isolates negatives for both mcr-1 & mcr-2. Lane 11: Negative control. **"B": Multiplex PCR for Int I 1-3 genes.** Lane 1: DNA ladder, Lane 2-10: Clinical isolates positive for integron-1, Lane 2&9: Clinical isolate positive for integron-2 (2000 bp band), Lane 7&8: Clinical isolate positive for integron-2 (788 bp band), Lane 11: Negative control. **"C": Variable regions of integron-I positive samples. "D": Variable regions of integron-II positive samples.**

DISCUSSION

As colistin represents the last resort of treatment options for CRE, resistance to it leads to more severe complications and increased mortality. Colistin-resistance is typically chromosomally mediated, however, plasmid-borne colistin-resistance mediated by *mcr* genes may lead to dissemination of pan-resistant GNB[17]. Integrons being capable of integrating, expressing, and disseminating gene cassettes, carrying resistance determinants, play a critical role in facilitating the MDR phenotype in bacteria[5]**.** This study was designed to survey the plasmid-encoded colistin-resistance *mcr-1* and *mcr-2* genes as well as the prevalence of class I-III integrons in colistin-resistant *Enterobacterales* isolated from hospitalized patients at KSMC, Riyadh, Saudi Arabia. The study also aimed to assess the performance of two commercially available BMD format; Sensititre™ GNX2F plate and Phoenix BD test for colistin susceptibility testing.

In the current study, *K. pneumoniae* represent the majority (97.6%) of colistin-resistance *Enterobacterales* isolates. The rest of the isolates were *K. oxytoca* (1.2%) and *Enterobacter cloacae* (1.2%). This agrees with a recent study from Saudi Arabia[18] and with the previous work of Manohar et al[19] and Das et al[20] but disagrees with Bir et al[21] findings who found that colistin-resistance reported in *E. coli* isolates was more than in *K. pneumoniae*.

The worldwide increase in colistin-resistance has been reported underlining the need for a robust and precise colistin susceptibility testing method[22]. International committees (EUCAST/CLSI) re-evaluated inconsistencies surrounding colistin AST, concluding that BMD should serve as the reference method for AST. Including BMD in routine antibiogram panels can be impractical in resource-poor settings because of its lengthy testing time, high cost, and extensive human resources. Several companies responded to this challenge, by bringing an easy-to use AST for colistin based on BMD method to the market. Most studies investigating colistin AST methods involved mainly colistin-susceptible GNB, while colistin-resistant isolates have hardly been tested[23]. In the current study, the accuracy of two commercially available colistin AST, Phoenix BD test and Sensititre test were evaluated against the reference BMD with 86 colistin-resistant isolates detected by Phoenix BD during routine testing.

Regarding the accuracy of colistin AST methods tested in the current study, CA of Phoenix BD test and the BMD method was 89.5% with an unacceptable rate of ME (10.5%). Some studies have reported a higher CA (95.24%) with acceptable rate of VME (3.17%) and ME (1.59%) and reported sensitivity, specificity, PPV and NPV of 95.56%, 95.24%, 97.73% and of 90.91%, respectively[24]**.** Others showed that Phoenix BD has unacceptable and inaccurate results with low specificity and sensitivity[25] and concluded that the Phoenix BD system, does not reliably distinguish colistin-resistant and colistin-susceptible strains.

Most of the few published studies have reported acceptable performance for Sensititre test for colistin testing**.** In the current study, CA of Sensititre test with the reference BMD method was 95.3% with no VME but with high ME rate of 4.6%. Sensitivity, specificity, PPV and NPV of the test were 100%, 95%, 56% and 100%, respectively. The measured CA in the current study for Sensititre was comparable to that reported in the study funded by European Society for Clinical Microbiology and Infectious Diseases (ESCMID)[26] but the later reported lower ME rate. Similarly, Mirza et al[27] have reported high CA for Sensititre but low EA with reference BMD, however, VME rate was just slightly above 3% and ME rate was acceptable. Other studies have reported less degree of agreement with the reference method and higher error rates[28].

In our study, colistin-resistant isolates were approximately 100% resistant to fluoroquinolones and penicillin combinations in a line with Arif et al[29]**.** Our isolates were generally susceptible to tigecycline, gentamycin and amikacin with variable degree which agree with the study of Moosavian and Emam in Iran[30] but disagree with the study of Pena et al in Madrid[31] who detected non-susceptibility to tigecycline in 36.5% of their strains.

The *mcr-1*-containing plasmids was found in many parts of the world among *Enterobacterales* and non-fermentative GNBs[1]. In recent years, reports of plasmid-mediated colistin resistance among *Enterobacterales* have raised concerns about the emergence of 'superbugs' that are resistant to this last resort of treatment. Clinical outbreaks involving colistin-resistant KPC-producing *K. pneumoniae* have been reported in the United States and Italy with worrying recurrence[32]. A recent report highlighted the presence of the *mcr-1* gene in *E. coli* isolated between 2012-2015 from Saudi Arabia, Bahrain, and United Arab Emirates[33]. The present study investigated the presence of plasmid-mediated *mcr-1* and *mcr-2* genes which detected *mcr-1* in only three (3.5%) isolates (all of them were *K. pneumoniae*), implying that colistin-resistance is mainly due to chromosomal elements. This agrees with Zafer et al[34]and Bir et al[21] who found *mcr-1* gene in only 5% and 6.7%, respectively of their isolates. The latter study reported multiple mutations in 10 genes responsible for lipopolysaccharide biosynthesis detected by whole genome sequencing of two non-mcr XDR *K. pneumoniae*.

More than half of the colistin-resistant studied isolates in our work were obtained from ICU patients (52.3%). The three *mcr-1* positive isolates were from patients admitted to ICU. Two of them have been

isolated from respiratory specimens while the third one was isolated from blood. None of the studied isolates in the current study harboured *mcr-2*, which is in line with study of Zafer et al[34]. *Mcr-2* was detected only in Belgium, indicating that it is probably dispersed *via* a different mechanism as *mcr-1* and are not transmitted from animal and environmental strains to human strains.

Previous studies show that clones of colistin-resistant *K. pneumoniae* in nosocomial setting show genome plasticity that facilitates the acquisition of diverse resistance determinants with multiple transposons and integrons found in these isolates[35]. The prevalence of these resistance determinants in colistin-resistant isolates is a serious threat for the evolution of pandrug resistance[35]. Screening of integons in our studied isolates, reveal high prevalence of integron I and II in colistin-resistant *K. pneumoniae* isolates (79.1% and 30.2%, respectively). Integron I was simultaneously detected in all strains positive for integron II, while integron III was not detected in our study. Our results are comparable to the results obtained in previous studies with prevalence of integron I and II ranges from 80-90% and 51.3%, respectively among *K. pneumoniae* isoltes[36], while disagree with other studies [37] which detected integron I in only about 40 % of tested *K. pneumoniae* strains. Other studies[38] reported a high prevalence of integrons (from 28.5% to 89.2%) in certain clinical populations. On the other hand, Zuhlsdorf study[39] reported low prevalence of integrons (13%) in enteric clinical isolates.

In our study, coexistence of integron I and II was detected in 30.2% of studied strains. This agreed with the study of Kargar et al[7] but was higher than the results reported by Kor et al[40] who found only one isolate carrying both integrons. The simultaneous existence of multiple integrons suggests their presence at different regions on the chromosome and plasmids of the isolate[9].

In the current study, gene cassettes were amplified in almost all the isolates positive for integron I and II implying the potential for multidrug resistance among the studied isolates. The most common gene cassette amplicons of integron I was single band at 800 bp and 2 bands at 800 + 1200 bp, while for integron II, it was at 1300 bp. These results were comparable to previous studies[37] where pattern of class I integron cassette amplicons were at 1500 bp, 700 bp + 1500 bp + 2000 bp, and 1500 bp + 2000 bp and integron II at 1500 bp and 2500 bp[36].

Kargar et al[7] reported significant associations between presence of integrons I and II and antimicrobial resistance. Our results revealed a significant association between the presence of intgron II and resistance SXT. This may be explained by the presence of gene cassettes which confer resistance to SXT in the variable region of integron II.

CONCLUSION

In conclusion, Sensititre test has an excellent CA with BMD with slightly high ME rates for colistin susceptibility testing. Phoenix BD results should be confirmed by another method due to high ME rate. Plasmid borne *mcr*- mediated colistin resistance plays a minor role in colistin-resistance among *K. pneumoniae* isolates. There is an alarming high prevalence of integron I and II among the MRD *Enterobacterales*. The strength of this study includes that it highlights the prevalence of integrons and *mcr*-mediated resistance in colistin-resistant *Enterobacterales* isolates. In also evaluates the accuracy of the colistin susceptibility tests on a large number of colistin-resistant *Enterobacterales* isolates. In addition, it evaluates a commonly used automated AST systems in modern labs; Phoenix BD test and another microdilution format, the Sensititre test. The limitation of this study includes that no colistin-sensitive CRE isolates were included. Future work will include whole genome sequencing of the studied isolates to explore the potential mechanisms conferring colistin resistance in these strains.

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Consent for publication

Not applicable

Availability of data and material

Data are available upon request

Competing interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article none.

REFERENCES

1. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 2016; 16: 161-168.

2. Yu Z, Qin W, Lin J, Fang S, Qiu J. Antibacterial mechanisms of polymyxin and bacterial resistance. Biomed Res Int. 2015; 2015:679109.
3. Dandachi I, Sokhn ES, Dahdouh EA, Azar E, El-Bazzal B, Rolain JM, Daoud Z. Prevalence and characterization of multi-drug-resistant gram-negative bacilli isolated from lebanese poultry: a nationwide study. Front Microbiol. 2018; 9:550.
4. Luo Q, Wang Y, Xiao Y. Prevalence and transmission of mobilized colistin resistance (mcr) gene in bacteria common to animals and humans. Biosafety Health. 2020; 2:71–8.
5. Sabbagh P, Rajabnia M, Maali A, Ferdosi-Shahandashti E. Integron and its role in antimicrobial resistance: A literature review on some bacterial pathogens. Iran J Basic Med Sci. 2021 Feb;24(2):136-142.
6. Ranjbar R, Taghipour F, Afshar D, Farshad S. Distribution of class 1 and 2 integrons among Salmonella enterica serovars isolated from iranian patients. Open Microbiol J 2019; 13:63- 66.
7. Kargar M, Mohammadalipour Z, Doosti A, Lorzadeh S, Japoni-Nejad. A High Prevalence of Class 1 to 3 Integrons Among Multidrug-Resistant Diarrheagenic Escherichia coli in Southwest of Iran. Osong Public Health Res Perspect 2014 5(4), 193e198
8. Turnidge J, Sei K, Mouton J. Polymyxin Susceptibility Testing and Breakpoint Setting. Adv Exp Med Biol. 2019;1145:117-132.
9. Clinical Laboratory Standard Institute (CLSI, 2020). Performance standards for antimicrobial susceptibility testing. M100, 30th ed.
10. Queipo-Ortuño MI, De Dios Colmenero J, Macias M, Bravo MJ, Morata P. Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis. Clin Vaccine Immunol. 2008 Feb;15(2):293-6.
11. Alghoribi MF, Doumith M, Upton M, Al Johani SM, Alzayer M, Woodford N. Complete Genome Sequence of a Colistin-Resistant Uropathogenic *Escherichia coli* Sequence Type 131 fimH22 Strain Harboring *mcr-1* on an IncHI2 Plasmid, Isolated in Riyadh, Saudi Arabia. Microbiol Resour Announc, 8 (18) (2019), pp. 1-3
12. Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, Hansen IM, Guerra B, Malorny B, Borowiak M, Hammerl JA, Battisti A, Franco A, Alba P, Perrin-Guyomard A, Granier SA, De Frutos Escobar C, Malhotra-Kumar S, Villa L, Carattoli A, Hendriksen RS. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5 for surveillance purposes. Euro Surveill. 2018 Feb;23(6):17-00672.
13. Machado E, Canton R, Baquero F, Galan J, Rollan A, Peixe L, et al, Integron content of extended-spectrum-b-lactamase-producing Escherichia coli strains over 12 years in a single hospital in Madrid, Spain, Antimicrob. Agents Chemother. 49 (5) (2005) 1823e1829.
14. Chan YH. Biostatistics 103: Qualitative Data – Tests of Independence. Singapore Med J.;2003, 44(10): 498-503.
15. Galen RS. Predictive values and efficiency of laboratory testing. Pediat J Clin North Am, 1982, 27:861-69.
16. Clinical Laboratory Standard Institute (CLSI, 2015). CLSI M52 document: Verification of Commercial Microbial Identification and Antimicrobial Susceptibility Testing Systems, 1st Edition, 2015.
17. MacNair CR, Stokes JM, Carfrae LA, Fiebig-Comyn AA, Coombes BK, Mulvey MR, Brown ED. Overcoming mcr-1 mediated colistin resistance with colistin in combination with other antibiotics. Nat Commun. 2018 Jan 31;9(1):458.
18. Garbati AM. Infection due to colistin-resistant Enterobacteriacae in criticallyill patients. J Infect Dev Ctries, 7 (10) (2013), pp. 713-719
19. Manohar P, Shanthini T, Ayyanar R*, et al*. The distribution of carbapenem- and colistin- resistance in Gram-negative bacteria from the Tamil Nadu region in India. *J Med Microbiol* 2017; 66: 874–883.
20. Das S, Roy S, Roy S*, et al*. Colistin susceptibility testing of gram-negative bacilli: better performance of Phoenix BD 2 system than E-test compared to broth microdilution method as the gold standard test. *Indian J Med Microbiol* 2020; 38: 58–65.
21. Bir R, Gautam H, Arif N, Chakravarti P, Verma J, Banerjee S, Tyagi S, Mohapatra S, Sood S, Dhawan B, Chaudhry R, Kapil A, Das BK, Das B. Analysis of colistin resistance in carbapenem-resistant *Enterobacterales* and XDR *Klebsiella pneumoniae*. Ther Adv Infect Dis. 2022 Feb 26;9.
22. Control ECfDPa. *Surveillance of antimicrobial resistance in Europe – Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2017*. Stockholm: ECDC, 2018.
23. Dafopoulou K, Zarkotou O, Dimitroulia E, Hadjichristodoulou C, Gennimata V, Pournaras S, Tsakris A. Comparative Evaluation of Colistin Susceptibility Testing Methods among Carbapenem-Nonsusceptible Klebsiella pneumoniae and Acinetobacter baumannii Clinical

Isolates. Antimicrob Agents Chemother. 2015 Aug;59(8):4625-30.

24. Shams N, AlHiraky H, Moulana N, et al.: Comparing quantitative and qualitative methods for detecting the in vitro activity of colistin against different Gram-negative bacilli. J Bacteriol Mycol. 2021, 8(5):1181.
25. Özyurt ÖK, Özhak B, Öğünç D, Yıldız E, Çolak D, Günseren F, Öngüt G. Evaluation of the BD Phoenix100 System and Colistin Broth Disk Elution Method for Antimicrobial Susceptibility Testing of Colistin Against Gram-negative Bacteria]. Mikrobiyol Bul. 2019 Jul;53(3):254-261.
26. Matuschek E, Åhman J, Webster C, Kahlmeter G. Antimicrobial susceptibility testing of colistin - evaluation of seven commercial MIC products against standard broth microdilution for Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter spp. Clin Microbiol Infect. 2018 Aug;24(8):865-870.
27. Mirza HC, Bıçakçıgil A, Liste Ü, Sancak B. Evaluation of a Commercial Broth Microdilution Panel for Colistin Susceptibility Testing of Clinical Isolates of Escherichia coli and Klebsiella pneumoniae. Clin Lab. 2021 May 1;67(5).
28. Yusuf E, van Westreenen M, Goessens W, Croughs P. The accuracy of four commercial broth microdilution tests in the determination of the minimum inhibitory concentration of colistin. Ann Clin Microbiol Antimicrob. 2020 Sep 14;19(1):42.
29. Arif A, Ullah A, Ullah O, and Zaman R. Identification of colistin resistance and its bactericidal activity against uropathogenic gram negative bacteria from Hayatabad Medical Complex Peshawar. Pak J Med Sci . v.38(4Part-II); Mar-Apr 2022.
30. Moosavian M and Emam N. The first report of emerging mobilized colistin-resistance (*mcr*) genes and ERIC-PCR typing in *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates in southwest Iran. Infect Drug Resist. 2019; 12: 1001–1010.
31. Pena I, Juan J Picazo, Carmen Rodríguez-Avial, Iciar Rodríguez-Avial. Carbapenemase-producing Enterobacterales in a tertiary hospital in Madrid, Spain: high percentage of colistin resistance among VIM-1-producing Klebsiella pneumoniae ST11 isolates. Int J Antimicrob Agents. 2014 May;43(5):460-4.
32. Sekyere JO, U. Govinden, L.A. Bester and S.Y. Essack. Colistin and tigecycline resistance in carbapenemaseproducing Gram-negative bacteria: emerging resistance mechanisms and detection methods. Journal of Applied Microbiology (2016): 121, 601--617.
33. Sonnevend A, Ghazawi A, Alqahtani M, Shibl A, Jama W, Hashmey R, Tibor Pal a. Plasmid-mediated colistin resistance in Escherichia coli from the Arabian Peninsula. International Journal of Infectious Diseases 50 (2016) 85–90.
34. Zafer MM, El-Mahallawy HA, Abdulhak A, Amin MA, Al-Agamy MH and Radwan HH. Emergence of colistin resistance in multidrug-resistant Klebsiella pneumoniae and Escherichia coli strains isolated from cancer patients. Annals of Clinical Microbiology and Antimicrobials volume 18, Article number: 40 (2019)
35. Arduino SM, Quiroga MP, Ramírez MS, Merkier AK, Errecalde L, Di Martino A, Smayevsky J, Kaufman S, Centrón D. Transposons and integrons in colistin-resistant clones of Klebsiella pneumoniae and Acinetobacter baumannii with epidemic or sporadic behaviour. J Med Microbiol. 2012 Oct;61(Pt 10):1417-1420. doi: 10.1099/jmm.0.038968-0. Epub 2012 Jun 21. PMID: 22723256.
36. Rizk D, El-Mahdy AM. Emergence of class 1 to 3 integrons among members of Enterobacterales in Egypt. Microbial Pathogenesis 112 (2017) 50e56
37. Manohar P, Shanthini T, Ekta P, Mahesan J B, et al. Colistin-Resistant Klebsiella pneumoniae: Prevalence of Integrons and Synergistic Out Turn for Colistin-Meropenem. Arch Clin Infect Dis. 2018 January; 13(1):e55099
38. Kang H, Jeong YS, Oh JY, Tae SH, Choi CH, Moon DC, et al., Characterization of antimicrobial resistance and class 1 integrons found in Escherichia coli isolates from humans and animals in Korea, J. Antimicrob. Chemother. 55 (2005) 639e644
39. Zuhlsdorf MT, Wiedemann B, Tn21-specific structures in gram-negative bacteria from clinical isolates, Antimicrob. Agents Chemother. 36 (1992) 1915e1921.
40. Kor SB, Choo Q, Chew CH, New integrin gene arrays from multiresistant clinical isolates of members of the Enterobacterales and Pseudomonas aeruginosa from hospitals in Malaysia, J. Med. Microbiol. 62 (2013) 412e420.