Infection and Drug Resistance

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ORIGINAL RESEARCH

Prevalence and Some Possible Mechanisms of Colistin Resistance Among Multidrug-Resistant and Extensively Drug-Resistant *Pseudomonas aeruginosa*

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Background and Aim: The emergence of colistin-resistant strains is considered a great threat for patients with severe infections. Here, we investigate the prevalence and some possible mechanisms of colistin resistance among multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Pseudomonas aeruginosa* (*P. aeruginosa*).

Methods: Antimicrobial susceptibility was performed using disc diffusion methods while colistin resistance was detected by agar dilution method. Possible mechanisms for colistin resistance were studied by detection of *mcr-1* and *mcr-2* genes by conventional PCR, detection of efflux mechanisms using Carbonyl Cyanide 3-Chlorophenylhydrazone (CCCP), studying outer membrane protein profile and Lipopolysaccharide (LPS) profile of resistant isolates.

Results: It was found that MDR and XDR represented 96% and 87% of the isolated *P. aeruginosa*, respectively, and colistin resistance represented 21.3%. No isolates were positive for *mcr-2* gene while 50% of colistin-resistant isolates were positive for *mcr-1*. Efflux mechanisms were detected in 3 isolates. Protein profile showed the presence of a band of 21.4 KDa in the resistant strains which may represent OprH while LPS profile showed differences among colistin-resistant *mcr-1* negative strains, colistin-resistant *mcr-1* positive strains and susceptible strains.

Conclusion: The current study reports a high prevalence of colistin resistance and *mcr-1* gene in *P. aeruginosa* strains isolated from Egypt that may result in untreatable infections. Our finding makes it urgent to avoid unnecessary clinical use of colistin.

Keywords: *Pseudomonas aeruginosa*, colistin resistance, *mcr-1*, *mcr-2*, *toxA* gene, XDR, MDR

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen, commonly found in environment such as soil, water, plants and hospital environment, with known intrinsic resistance to many antimicrobials and the ability to cause life-threatening infections. It is considered the second common cause of sepsis in intensive care units (ICUs) and can cause ventilator-associated pneumonia, wound infections and urinary tract infections (UTI). Many studies reported the increase of mortality and morbidity of infections associated with *P. aeruginosa,* especially those showing multi-drug resistance patterns.[1–3]

The emergence of multidrug-resistant (MDR) or extensively drug-resistant (XDR) or pandrug-resistant (PDR) *P. aeruginosa* becomes a significant public health problem that can lead to delayed antimicrobial therapy or its failure and

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the increase in the mortality rate especially with the appearance of carbapenem-resistant *P. aeruginosa*. So, attention is required because these resistant strains may show resistance to all available antimicrobials or showed susceptibility only to toxic ones such as colistin or polymyxins leaving no choices for the health-care team in the treatment of severe infections associated with MDR *P. Aeruginosa*.[4]

Recently, emergence of resistance to polymyxins was observed among certain species of Enterobacteriaceae such as *K. pneumoniae, E. coli, Enterobacter aerogenes* and *Enterobacter cloacae* due to its wide use to control infections in veterinary medicine. Colistin resistance has become a major challenge for the treatment of life-threatening infections especially with the co-existence of *mcr-1* genes with other multiple drug resistance genes as ESBL, MBL, NDM genes with the possibility of the emergence of pan-drug resistance.[5,6]

Colistin, known as polymyxin E, is one member of a family of cationic polypeptide known as polymyxins. This antibiotic family is characterized by the presence of a lipophilic fatty acyl side chain. Nowadays, colistin is reintroduced in medical therapy and considered the last resort for the treatment of severe infections caused by MDR and XDR stains. In general, the action of polymyxins on bacteria depends mainly on the electrostatic interaction between the positively charged antibiotic and the negatively charged phosphate group of lipid A localized on the outer membrane after its binding, it diffuses through the outer membrane, periplasmic space and interact with the inner membrane. Polymyxins cause destabilization to the outer membrane, pore formation, increase permeability, leakage to cytoplasmic content followed by cell lysis.[7]

Colistin resistance mainly occurs due to the chemical modification by the enzymatic addition of phosphoethanolamine at the 4′- phosphate group of the lipid A moiety of the lipopolysaccharide decreasing the net-negative charge of the outer membrane resulting in decreasing polymyxin affinity. Resistance to colistin may be resulted from chromosomally encoded mutation as reported in *K. pneumoniae* or the horizontal transfer of resistance by means of plasmid carrying colistin-resistant gene (*mcr-1*).[8–11]

The emergence of colistin resistance in various countries in Asia, Europe and some countries in Africa has become one of the global concerns. As, colistin resistance dissemination indicates its ability to transfer horizontally by conjugative plasmids or vertically by chromosomal mutation.[12,13] Also, being colistin one of the last lines of treatments to serious infections, making the emergence of colistin resistance isolates threatening the world by the appearance of untreatable infectious diseases.[14] Detection of colistin resistance in Egypt, which is a country known by its high burden of infectious diseases and the presence of low or no restriction on the antimicrobial use in both veterinary and medicine, indicating the emergence of untreatable diseases in our area due to the possibility of transferring colistin resistance to highly resistant bacteria.[15]

In this study, we investigate the prevalence of colistin resistance among MDR and XDR *P. aeruginosa* isolated from patients suffering from a variety of infections in the intensive care unit (ICU) of Minia university hospital in Egypt.

Materials and Methods

Collection of Isolates

One hundred-seventy five clinical samples of different sources of infections were collected from patients admitted to ICU in Minia university Hospital, Minia, Egypt as part of routine hospital-laboratory procedures. All clinical samples were cultured on trypticase soy agar (Lab M, UK) at 37°C and 42°C for 24 hrs. One colony was sub-cultured on MacConkey agar plates and cetrimide agar. Isolated colonies were further identified according to colony morphology, lactose fermentation, biochemical reactions (including sulphide–indole motility, catalase, triple sugar iron, urease and oxidase tests), ability to grow on cetrimide agar and to grow at 42°C.[16] *P. aeruginosa* colonies were purified by streaking, and pure colonies were stored at 4°C.

Antibiotic Susceptibility Tests

Antibiotic Susceptibility by Kirby-Bauer Disc Diffusion Method

The antibiotic susceptibility against different classes of antibiotics was tested by the Kirby-Bauer disc diffusion method.[17] Antibiotic discs used were amoxicillin/clavulanic (AMC) (20/10 µg), ampicillin/sulbactam (SAM) (20 µg), meropenem (MEM) (10 µg), imipenem (IPM) (10 µg), cefepime (FEB) (30 µg), cefoperazone (CEP) (75 µg), polymyxin B (PB) (300 µg), ciprofloxacin (CIP) (5 µg), levofloxacin (LEV) (5 µg), gentamicin (CN) (10µg), ceftazidime (CAZ) (30 µg), tigecycline (TGC) (15 µg), amikacin (AK) (30 µg), tobramycin (TOB) (10 µg), aztreonam (ATM) (30 µg), piperacillin (PRL) (30 µg), carbenicillin (CAR) (100 µg) (Oxoid; Basingstoke, UK). Isolates were classified as sensitive,

intermediate and resistant according to inhibition zones' interpretation standards of Clinical Laboratory standards Institute (CLSI) 2018.[18]

MIC Determination of Colistin Antibiotic

Agar dilution method on Muller-Hinton agar was used to determine the colistin minimum inhibitory concentration.[19] Resistance to colistin was considered if the MIC is ≥4μg/mL according to the standard guidelines of CLSI.[18]

According to the results of antibiotic susceptibility, isolates were classified to MDR, XDR and PDR according to the criteria previously reported.[20]

Combined Disc Diffusion Test (CDT)

All colistin-resistant isolates (MIC ≥4) were tested using 100 mM EDTA (Sigma-Aldrich; St.268 Louis, MO, USA) to inhibit the *mcr-1* activity as this concentration showed no antimicrobial activity. The bacterial strains were cultured on Muller-Hinton agar (Lab M, UK) on which three discs were used. One disc was saturated with 10 µL of 100 mM EDTA to insure no inhibition of the bacterial growth by the used concentration of EDTA. The other two discs were 10µg colistin disc and 10 µg colistin plus 10 µL 100 mM EDTA disc. The isolates were observed for an increase of ≥3mm in the inhibition zone diameter of the colistin/EDTA disc comparing to the colistin disc.[21]

Alteration of Zeta Potential

The *mcr* genes encode phosphoethanolamine transferases enzymes which attach enzymatically a phosphoethanolamine (PEtN) moiety to the lipid A of the outer membrane of Gram-negative bacteria leading to reduction in its net negative charge conferring the colistin resistance.[22]

The bacterial cells have been allowed to grow in the presence and absence of 80 µg/mL EDTA. Then, the bacterial suspension was centrifuged at 5000 rpm for 5 min at 5°C then pellets were washed twice, after that pellets were suspended in 2 mL of sterile 1 mM NaCl solution adjusted to 0.5 McFarland standard solution turbidity. Samples were diluted to 1:4 using 1mM NaCl. Zeta potential was determined in 2 mL of the diluted sample. Alterations of Zeta potential induced by EDTA were calculated from the Zeta potential ratio (RZP=ZP+EDTA/ZP-EDTA), where ZP+EDTA and ZP-EDTA correspond to Zeta potential values obtained for bacterial suspensions grown in the presence or absence of 80µg/mL EDTA, respectively. RZP of ≥ 2.5 value considered as criteria for the identification of *mcr-1* positive strains.[21]

DNA Extraction

The DNA template was extracted from an overnight culture of *P. aeruginosa* as previously described.[23] A suspension of bacterial pellet was boiled for 10 min, then, centrifuged. Supernatant was used directly in the PCR assay.

PCR Analysis of the Tested Genes

Exotoxin A is an important virulence factor (a cytotoxic agent) of *P. aeruginosa* in clinical infections. This factor inhibits protein biosynthesis leading to great tissue and organ damage. The *toxA* gene, an inherent genetic sequence located on the *P. aeruginosa* chromosome, is used for *P. aeruginosa* confirmation by PCR.

PCR was performed in a total volume of 25 µL containing 1X PCR buffer, 1 µmol/L of each primer, 1 µL of genomic DNA (approximately150 ng), 200 µmol/L of dNTPs mix, 2 mmol/L of MgCl2, and 0.05 U/µL Taq DNA polymerase. PCR amplifications were performed for *toxA* FW:CTGCGCGGGTCTATGTGCC, RV:GATGCTGGAC GGGTCGAG in an automated thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: 30 cycles of 1 min at 94°C, 1.5 min at 63°C, and 1 min at 72°C.[24]

The genes *mcr-1* and *mcr-2* were assayed by conventional PCR technique using the following primers: *mcr-1* FW (5′-AGTCCGTTTGTTCTTGTGGC-3′), RV (5′-AGAT CCTTGGTCTCGGCTTG-3′) and *mcr-2* Fw (5′-ATGAC ATCACATCACTCTTGG-3′), Rv (5′-TTACTGGATAAAT GCCGCGC-3′).[25,26] The technique conditions were 34 cycles of 95°C for 1 min, 58°C for mcr-1 and 52°C for 30s, 72°C for 1min followed by final extension of 72°C for 5 mins.

Determination of Efflux Pumps Inhibition by MIC Reduction Using Efflux Pump Inhibitor (CCCP)

The agar dilution method was used for the determination of MICs using the Cation-adjusted Mueller-Hinton broth (Sigma-Aldrich, St Louis, USA). The MICs of CCCP (EPI) and colistin were determined for the tested isolates. Sub-MIC of CCCP was used in determining its effect on colistin MIC; the concentration of CCCP (0.5× MIC) was constantly kept at the MIC concentrations stated above whilst that of the antibiotic were serially increased. The MICs of the isolates to colistin in the absence and presence of CCCP were determined using a sub-MIC of CCCP (final concentration of 10 mg/L) as

already described.[27] The resulting MIC fold changes after the addition of CCCP were calculated as the ratio of the CCCP-free antibiotic's MIC level to that of the CCCP-added antibiotic. As previously described by Osei Sekyere, Amoako[28] who reported that the positive criterion for the presence of efflux pumps in isolates was a ≥8-fold decrease in colistin MIC after adding CCCP.

Outer Membrane Protein Pattern

A single colony of the tested *P. aeruginosa* isolates was cultured in 5 mL of LB broth at 37°C for 2 days with shaking at 200 rpm. Cells were centrifuged at 8000 rpm for 5 mins. Bacterial pellets were suspended in 1 mL of lysis buffer (0.05 M Tris HCL, 2% SDS, 10% glycerol), heated at 95°C for 10 mins. Then, the samples were centrifuged for 10.000 rpm for 30 min. About 50 µL of extracted protein was mixed with sample buffer (4 mL deionized water, 1 mL of 0.5 M Tris HCL, 1.6mL 10% SDS, 0.4 mL 2-mercaptoethanol, 0.2 mL of 1% (w/v) Bromophenol blue) (1:1) and separated by 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE).[29]

Lipopolysaccharide SDS-Polyacrylamide Gel Profile for Colistin Sensitive and Colistin-Resistant Isolates

LPS of the tested isolates were extracted and purified by hot aqueous-phenol method using Westphal, Jann[30] and analyzed the purified material using SDS-PAGE, followed by carbohydrate-specific silver staining.[31]

Results

Pseudomonas aeruginosa Isolation and Antibiotic Susceptibility

Out of 175 samples collected from patients suffering from different infections, 75 samples (42.8%) were positive phenotypically for *P. aeruginosa* and positive for *toxA* gene.

Antimicrobial susceptibility testing revealed that the isolated *P. aeruginosa* were completely resistant to amoxicillin/clavulanic acid and high resistance was observed against ampicillin/sulbactam (68%), ceftazidime (63%) and azetreonam (60%). Moderate resistance was observed against both tobramycin and tigecycline (50% each). Furthermore, low resistance was shown against imipenem (6%) and meropenem (5.3%) (Figure 1). According to the antibiotic susceptibility results the resistant isolates were classified to MDR (96%), XDR (87%) and no isolate was classified as PDR. In addition, it was found that out of 75 isolates, 16 isolates (21.3%) showed resistance to colistin antibiotic with MIC ≥ 4µg/mL (ranged from 8 to 256 µg/mL).

Determination of *Mcr-1* and *Mcr-2* Genes

Mcr-1 gene was detected phenotypically in the colistin-resistant isolates by CDT where the differences between the diameters of inhibition Zones of colistin/EDTA and

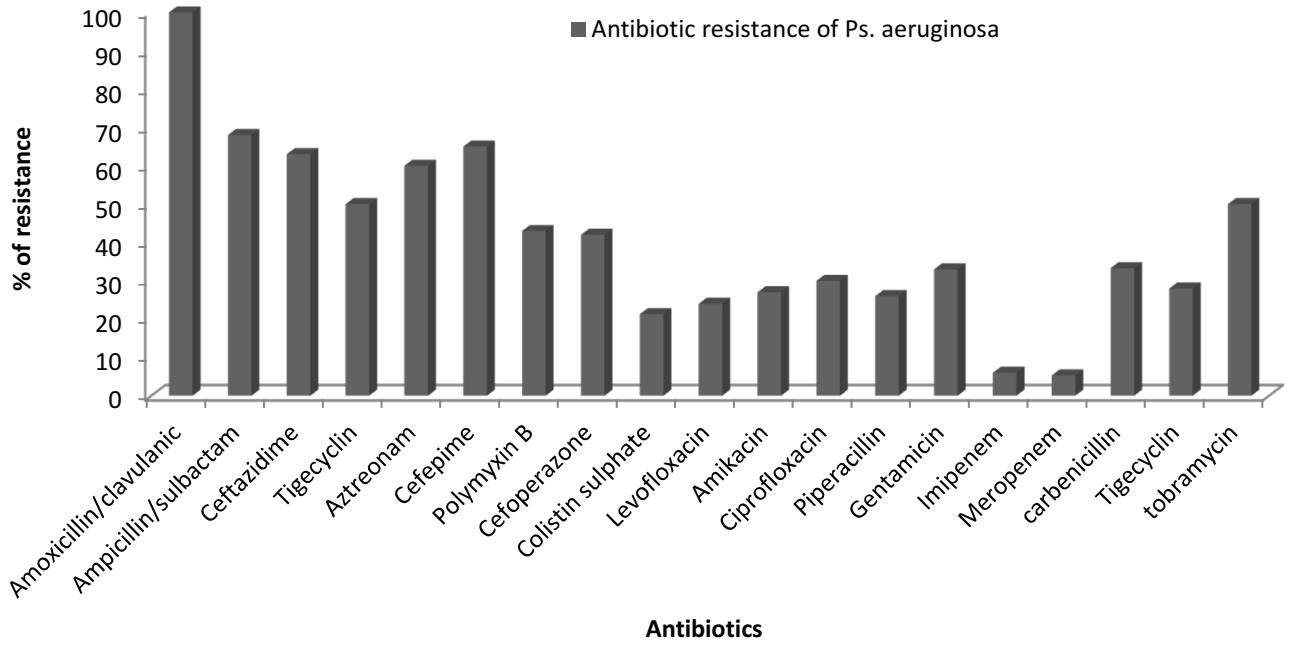

Figure 1 Antibiotic resistance pattern of all isolated *P. aeruginosa* isolates.

colistin discs were measured to be ≥ 3mm. The results showed that 6 isolates (37.5%) showed an increase in the diameter of the colistin/EDTA disc by 3 to 10 mm in comparison to colistin disc alone (Figure 2).

Alteration of Zeta Potential

On the other hand, alteration of Zeta potential assay was held as a phenotypic detection to MCR genes, but results showed no significant change in the zeta potential except in 2 isolates.

Detection of Resistance Genes

The genetic detection of *mcr* genes using conventional PCR technique revealed that 8 (50%) isolates were positive for *mcr-1*, 6 of them were positive for CDT, while 100% (16 isolates) were negative for *mcr-2*.

Antibiotic Susceptibility of Colistin-Resistant Isolates

The susceptibility of the colistin-resistant isolate against other antibiotics was determined by Kirby-Bauer disc diffusion method, the results showed that 100% of isolates were resistant to Amoxicillin/clavulanic, while resistance to Ampicillin/sulbactam, Cefepime and Tobramycin was 78.12%, 71.87% and 68.75% respectively. The most effective drugs were meropenem, imipenem and ciprofloxacin (Figure 3).

Determination of Efflux Pumps Inhibition by MIC Reduction Using CCCP

By studying the effect of 0.5 MIC of CCCP on the MIC colistin, it was found that only 3/16 isolates (P6, P8 & P16) (18.75%) showed a reduction in the MICs of colistin ≥ 8 fold (Table 1) in the presence of CCCP. From previous results, the isolate no. 16 was found to have efflux mechanism and *mcr-1* gene.

Outer Membrane SDS-PAGE Profile

Table 2 and Figure 4 show that five bands with molecular weights of 66.7, 56.06, 47.8, 40.18 and 23.6 KDa were stable in sensitive and resistant isolates while one band with a molecular weight of 21 KDa was found only in colistin-resistant strains which were P1 (*mcr-1* positive) and P12 (*mcr-1* negative).

Lipopolysaccharide (LPS) SDS-PAGE

Lipopolysaccharide silver-stained SDS-PAGE showed that colistin-resistant *mcr-1* negative isolates (P3, P6 and P10) showed no LPS bands pattern (O-antigen repeats or LPS core) that revealed the possibility of their loss and the

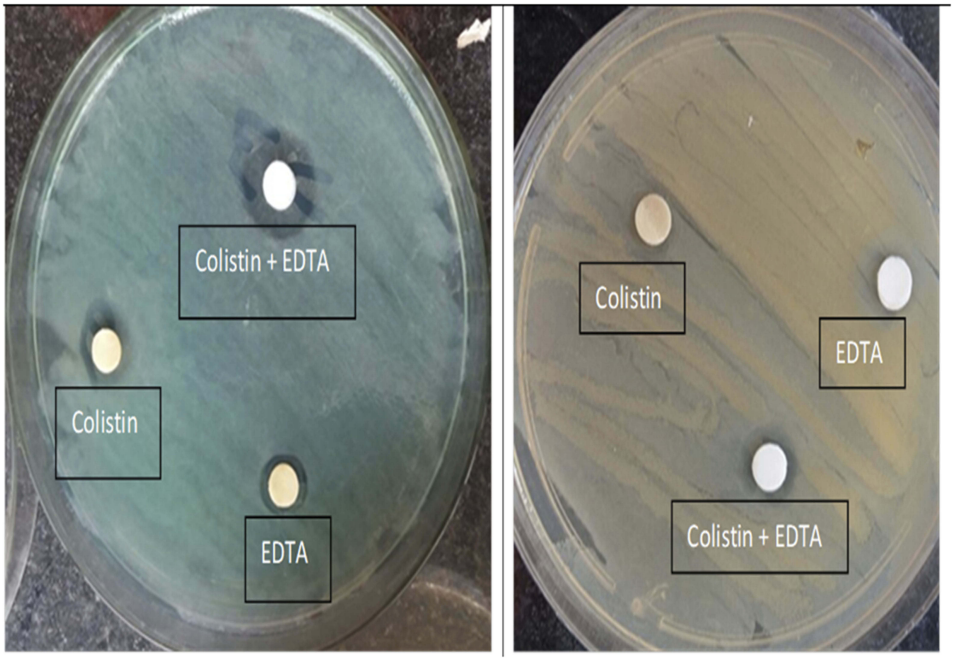

Figure 2 Phenotypic detection for *mcr* positive isolates by combined disc diffusion test (CDT). (**A**): *mcr-1* positive strain showed an increase in the zone diameter of discs with colistin and EDTA ≥ 3mm in comparison to colistin alone. (B): mcr-1 negative isolate showed slight change (1 mm) in the inhibition zone diameter of colistin and EDTA disc in comparison to colistin alone.

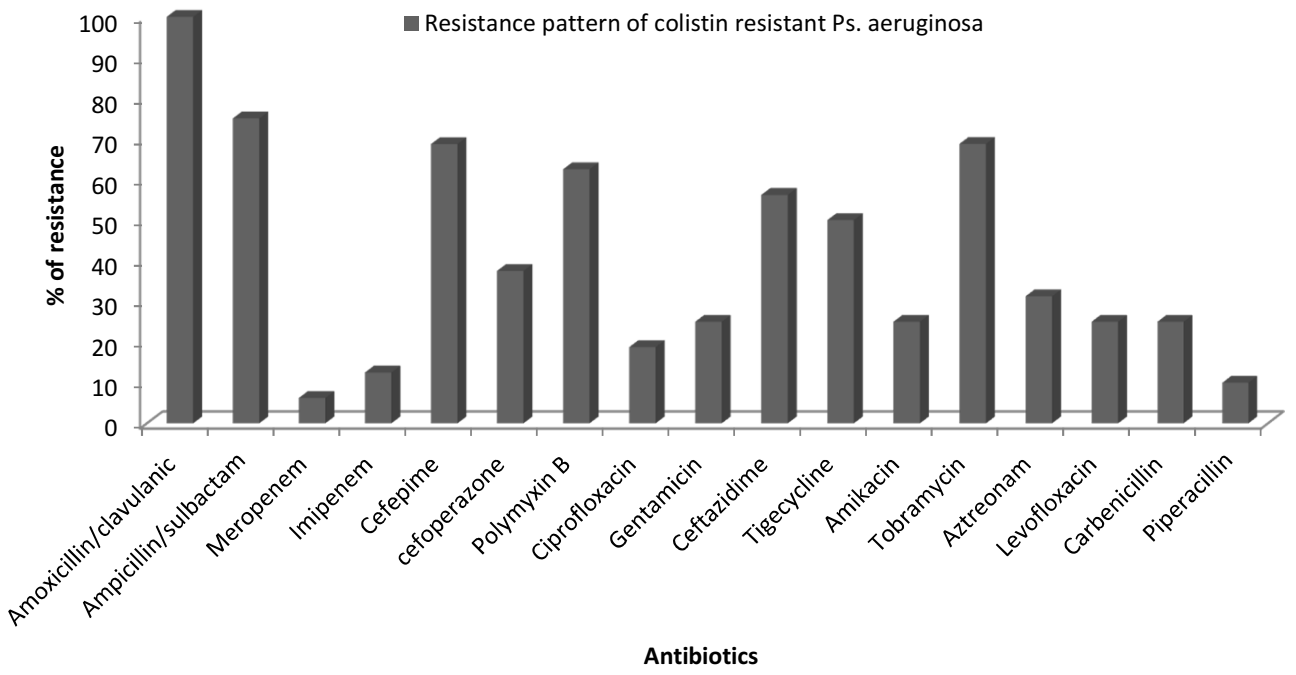

Figure 3 Antibiotic resistance pattern of colistin-resistant isolates.

resistance of these isolates to colistin. On the other hand, Colistin-resistant *mcr-1* positive strain showed O-antigen repeats (Figure 5, Lane 5) that differs from O-antigen repeats pattern of Colistin sensitive strain (Figure 5, Lane 4) while both showed LPS core. These results may indicate the presence of modified LPS in the *mcr-1* positive strain.

Discussion

Recently, multidrug-resistant pathogenic bacterial strains appear where most of the available antibiotics are not effective against them.[6,32–36] The polymyxins considered the last resort for treatment of multi-drug resistant bacterial infections, so studying the emergence of colistin-resistant was a must. Polymyxins showed their activity through their electrostatic interaction between them and the negatively charged moieties on the lipid A of Gram-negative bacteria resulting in the destabilization of the outer membrane and the leakage of the cytoplasmic content and lysis.[37,38]

It was found that the most common cause of polymyxin resistance is LPS modification by the addition of 4-amino- 4-deoxy-L-arabinose (Lara4N) and phosphoethanolamine (encoded by *mcr*-type genes) or galactosamine to lipid A of LPS core. As a result, a decrease in the net-negative charge of phosphate residues affects the affinity of polymyxin to the membrane or due to the effect of two-component regulatory systems (TCSs) pmrA/pmrB and phoP/phoQ.[39]

In our study, we detected colistin resistance according to the results of MICs followed by their testing for the presence of *mcr-1* phosphoethanolamine transferase using phenotypic methods and the detection of *mcr-1*gene. Phenotypic methods depend on that *mcr-1* phosphoethanolamine is zinc metalloprotein. So, any decrease in zinc will decrease MICs of colistin in isolates positive for *mcr-1*. Being *mcr-1* encoding enzyme, a zinc metalloprotein permits using EDTA as a metal chelator to decrease zinc in media and affect colistin MICs and the zeta potentials of *mcr-1* positive isolates.[40]

Our study showed high prevalence of *P. aeruginosa* (42.8%). MDR *P. aeruginosa* corresponded to 96% of total isolates and 87% was XDR. High prevalence of colistin-resistant *P. aeruginosa* (21.3%) was detected which may be a result of insufficient infection control measures and misuse of bactericidal antibiotics in the intensive care units of our country hospitals. In addition, Colistin is widely used in our countries in the growth promotion of food-producing animals, especially in poultry industry while carbapenems used in emergency cases.[15] So, carbapenems showed observable activity against the tested organisms in comparison to colistin. On the other hand, our results were observed to be higher than those reported by Liassine et al[25] who reported that one isolate of 300 isolates of different bacterial species

Table 1 Colistin-Resistant Isolates, Some Possible Mechanisms of Resistance to Colistin and Their Susceptibility to Other Antibiotics

Isolates*	Colistin MIC µg/mL	*mcr1/ mcr-2*	CDT[a]	RZP[b]	MIC of Colistin in Presence of 0.5 MIC CCCP (µg/mL) (Fold Change)[c]	MDR, XDR or PDR	Antibiotics Showing Activity on the Tested Isolates
P1	8	+/–	+	1.07	8 (no change)	MDR	MEM, TGC, CIP, AK, CAR, PRL
P2	16	+/–	+	0.8	8 (2 folds)	MDR	MEM, TGC, CAR, CN, PRL
P3	256	–/–	-	0.7	128 (2 folds)	XDR	MEM, IMP, AK
P4	256	+/–	+	0.57	128 (2 folds)	MDR	TGC, CIP, MEM, PRL, AK
P5	256	+/–	+	1.08	256 (no change)	MDR	MEM, TGC, CAR, CN, PRL, CIP
P6	128	–/–	-	1.035	16 (8 folds)	MDR	MEM, TGC, CAR, AK, PRL
P7	128	–/–	-	1.28	64 (2 fold)	MDR	MEM, TGC, CAR, CN, PRL, ATM
P8	256	–/–	-	0.99	16 (16 folds)	XDR	MEM, TGC
P9	256	+/–	+	1.07	64 (4 folds)	MDR	TGC, CIP, MEM, PRL, AK
P10	32	–/–	-	1.08	16 (2 folds)	MDR	TGC, CIP, MEM
P11	128	–/–	-	1.055	64 (2 folds)	MDR	TGC, CIP, MEM, IMP, AK, CN
P12	256	–/–	-	1.48	256 (no change)	MDR	TGC, CIP, AK, LEV
P13	64	+/–	+	2.7	64 (no change)	MDR	TGC, CIP, MEM, PRL, AK
P14	256	–/–	-	0.935	128 (2 folds)	MDR	TGC, CIP, MEM, PRL, AK
P15	128	+/–	-	1.57	32 (4 folds)	XDR	MEM, PB
P16	64	+/–	-	2.9	8 (8 folds)	MDR	TGC, CIP, MEM, PRL, AK, CN

Notes: *All isolates were positive for toxA gene; [a]Combined disc diffusion test, [b]Zeta potential Ratio= ZP_{+EDTA}/ZP_{-EDTA}, [c]strains were considered as positive for efflux if fold change ≥ 8 folds. MDR: multi-drug resistant: nonsusceptible to ≥ 1 agent in ≥ 3 antimicrobial categories. XDR: nonsusceptible to ≥ 1 agent in all but ≤ 2 antimicrobial categories.

Abbreviations: CN, Gentamicin; AK, Amikacin; PB, ploymxin B; MEM, meropenem; TGC, Tigecycline; CEP, cefeperazone; CIP, ciprofloxacin; IPM, imipenem; PRL, piperacillin; LEV, levofloxacin; CAR, Carbenicillin.

Table 2 Molecular Weights and Amount % of Extracted Outer Membrane Proteins of Colistin Resistant and Colistin Sensitive *P. Aeruginosa*

Lanes:	M		Colistin Resistant Isolates				Colistin Sensitive Isolates					
			R1 (P1)		R2 (P12)		S1		S2		S3	
Rows	Mol.w.	Amount	Mol.w.	Amount	Mol.w.	Amount	Mol.w.	Amount	Mol.w.	Amount	Mol.w.	Amount
r1	170	5.326371										
r2	125	9.451697										
r3	81	10.44386	105	27.13755			105.75	7.895928	105.5	61.66282	105.55	37.32484
r4	62	9.112272	66.75	3.475836	66.071	3.128621	66.071	5.723982	66.07	3.903002	66.714	41.65605
r5			54.607	5.390335	54.686	36.3847	54.643	11.04072	54.693	6.30485	56.857	5.070064
r6	53	7.650131	47.874	3.289963	47.848	10.39397	47.839	11.04072	47.806	10.46189	47.774	9.312102
r7	43	8.381201	40.186	5.130112	40.174	10.16686	40.195	14.47964	40.163	7.459584	40.186	2.267516
r8	32	11.69713							31.364	5.034642		
r9	25	10.86162	23.684	7.843866	23.673	6.31924	23.177	5.904977	23.684	5.17321	23.886	4.280255
r10			21.456	6.003717	21.21	7.31205						
r11	17	11.93211										
r12	14	15.22193					14.482	43.8914				

was identified as *P aeruginosa* showing resistance to colistin and harboring *mcr-1* gene.

Combined disc diffusion test (CDT) and the alteration of zeta potential induced by EDTA were used as phenotypic methods[41,42] for the detection of *mcr-1* gene. The results showed that no isolates were positive for *mcr-2* and 8 (50%) isolates of colistin-resistant isolates were *mcr-1* positive while 2 isolates of these isolates showed RZP > 2.5. Out of 8 *mcr-1* positive isolates, 6 isolates were positive for CDT while two *mcr-1* positive (strain No. P15 and P16) were negative for CDT which may be due to co-production of additional mechanism of colistin resistance that interferes with the effect of EDTA.[21] As, it was found that isolate no. P16 (*mcr-1* positive and CDT

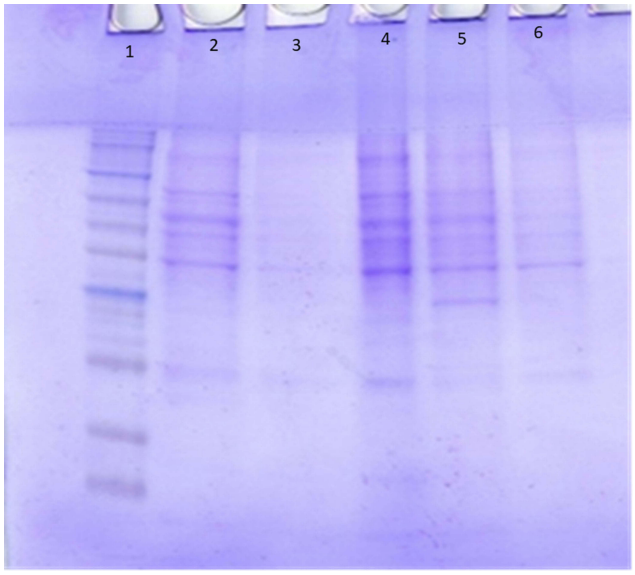

Figure 4 Outer membrane SDS-PAGE of colistin resistant and sensitive strains. Lane 1: Protein Marker, Lane 2 and Lane 3: colistin-resistant strains (P1 & P12), Lanes 4–6: colistin sensitive strains.

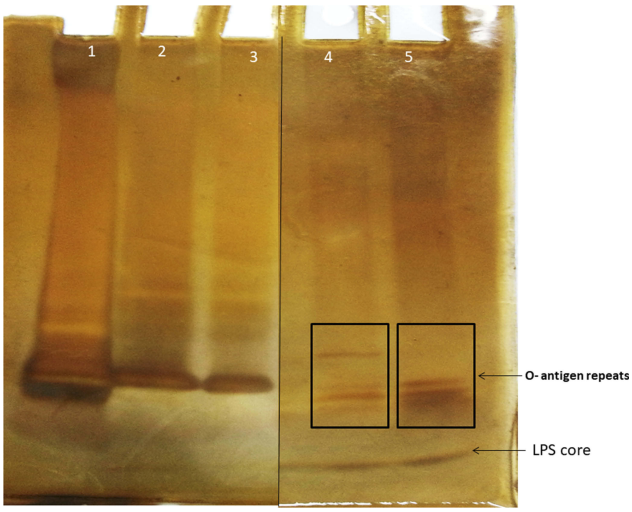

Figure 5 LPS bands pattern. Lanes 1, 2 & 3: colistin-resistant *mcr-1* negative strains (P3, P6 & P10, respectively), Lane 4: Colistin sensitive strains and Lane 5: Colistin-resistant *mcr-1* positive strain (P1). O-antigen repeats are boxed and arrow refers to LPS core.

negative) was positive for efflux.[21,43–45] In addition, colistin-resistant isolates that were negative for *mcr-1* may have mutations due to the long-term use of antimicrobials.

Furthermore, we tested colistin-resistant isolates for the presence of efflux mechanisms using CCCP (an efflux pump inhibitor) and the difference in outer membrane protein and LPS SDS-PAGE profile among sensitive and resistant isolates. Our results revealed the presence of efflux mechanism among 3 isolates while one of them was *mcr-1* positive. Outer membrane protein profile showed one band with a molecular weight of 21 KDa in the resistant isolates P1 (*mcr-1* positive) and P12 (colistin-resistant *mcr-1* negative). In addition, it was found that colistin-resistant *mcr-1* negative strains showed no LPS bands pattern (O-antigen repeats or LPS core) but *mcr-1* positive (P1) and colistin sensitive isolates showed LPS core but different O-antigen repeats pattern. Machado et al[20] studied the role of efflux pump in colistin resistance in *Acinetobacter baumanni* and found that efflux activity contributes to the heteroresistance of *A. baumanni* in absence of mutation. Marjani et al[43] showed that 22.5% of the isolated *P. aeruginosa* were resistant to colistin which is close to our results and more than 50% of colistin-resistant isolates were positive for efflux pumps.

Although the exact mechanism of bacterial killing by colistin or polymyxins is not clearly known, it is known that their binding to the positively charged peptides and the negatively charged Lipid A is a critical step. So, we tested their LPS SDS-PAGE profile and a significant difference among the tested strains was observed. In a study done by Moffatt et al,[46] it was reported that the loss of LPS resulted in the emergence of *A. baumanii* colistin resistant which occurs due to the inactivation of lipid A biosynthesis genes (*lpxA, lpxC*, or *lpxD*). Outer membrane protein patterns showed the presence of a band of molecular weight which is 21 KDa in colistin-resistant isolates which may correspond to OprH according to that reported by Nicas and Hancock[47] who reported that OprH expression plays a role in the resistance of Pseudomonas to polymyxins and EDTA because OprH replaces divalent cations in the outer membrane resulting in the blocking of polycationic antibiotic uptake. The previous finding may explain why strain no. P1 (*mcr-1* positive) was negative for CDT.

Conclusions

The present study showed a high prevalence of MDR and XDR *P. aeruginosa* showing colistin resistance among patients admitted to ICU suffering from different infections. Also, it showed the presence of different mechanisms that can result in colistin resistance. This indicates the urgent need of changing the antibiotic-treatment strategies for both humans and animals.

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Disclosure

The authors report no conflicts of interest in this work.

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