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Major Article

Plasmid-mediated *mcr-1* gene in *Acinetobacter baumannii* and *Pseudomonas aeruginosa*: first report from Pakistan

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Abstract

Introduction: The increased use of colistin against infections caused by *Acinetobacter baumannii* and *Pseudomonas aeruginosa* has resulted in colistin resistance. The purpose of this study was to detect plasmid-mediated *mcr-1* gene in colistin-resistant *A. baumannii* and *P. aeruginosa* isolates. **Methods:** A total of 146 clinical isolates of *A. baumannii* (n = 62) and *P. aeruginosa* (n = 84) were collected from the four largest tertiary care hospitals in Peshawar, Pakistan. All bacterial isolates were phenotypically screened for multidrug resistance using the Kirby–Baur disc diffusion method. The minimum inhibitory concentration (MIC) of colistin in all isolates was phenotypically performed using dilution methods. *mcr-1* gene was detected through polymerase chain reaction and the nucleotide sequence of amplicon was determined using Sanger sequencing. **Results:** Approximately 96.7% *A. baumannii* and 83.3% *P. aeruginosa* isolates were resistant to multiple antibiotics. Colistin resistance was found in 9.6% (6/62) of *A. baumannii* and 11.9% (10/84) of *P. aeruginosa* isolates. Among 16 colistin resistant isolates, the *mcr-1* gene was detected in one *A. baumannii* (1.61% of total isolates; 16.6% of colistin resistant isolates) and one *P. aeruginosa* strain (1.19% of total isolates; 10% of colistin resistant isolates). Nucleotide BLAST showed 98–99% sequence similarity to sequences of the *mcr-1* gene in GenBank. **Conclusions:** Our study reports, for the first time, the emergence of plasmid-mediated *mcr-1*-encoded colistin resistance in multidrug resistant strains of *A. baumannii* and *P. aeruginosa*. Further large scales studies are recommended to investigate the prevalence of this mode of resistance in these highly pathogenic bacteria.

Keywords: Multiple drug resistance. Colistin resistance. *mcr-1*. *Acinetobacter baumannii. Pseudomonas aeruginosa.*

INTRODUCTION

The rapidly rising health concerns due to emerging multiple drug-resistant (MDR) Gram-negative bacterial species, mainly *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and members of Enterobacteriaceae, and the unavailability of new antibiotics, pose an urgent need to address the issue on a global scale[1]. MDR-related concerns have been highlighted in February 2017 by the World Health Organization (WHO), following the inclusion of carbapenem resistant *A. baumannii* and *P. aeruginosa* in the list of the most virulent pathogens for which new effective antibiotics and therapies are urgently required[2,3].

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A. baumannii and P. aeruginosa are key ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp*). that cause hospital-related infections such as bacteremia, urinary tract infections, skin infections, soft tissue infections, and ventilator-associated infections at a variety of anatomical sites primarily in already ill and immune-deficient individuals[4-7]. These critical pathogens are becoming resistant to almost all important classes of antibiotics such as carbapenems, aminoglycosides, beta-lactams, and fluroquinolones, thus leaving behind no appropriate treatment option[8]. This issue is extremely serious for clinicians to treat infections caused by MDR *A. baumannii* and *P. aeruginosa* Gram-negative bacteria. Hence, WHO has included colistin in the list of "last-resort antibiotics" to be used against these emerging superbugs[9].

Colistin, also called as Polymyxin E, is an old antibiotic that belongs to the polymyxin family. It was first introduced in the 1950s, but owing to its harmful effects on human health affecting

primarily renal functions, its use was banned by many countries. However, when the remarkable spread of Carbapenem-resistant *A. baumannii*, *P. aeruginosa,* and Enterobacteriaceae members occurred, colistin was reintroduced in clinical settings after 60 years to treat infections caused by these bacteria[10,11].

Numerous mechanisms are responsible for colistin resistance in Gram-negative bacteria. The mutations occur in genes and is the most common mechanism through which Gram-negative bacteria develop resistance against colistin[12-14]. However, the resistance developed owing to changes in the lipo-polysaccharide layer, by adding phospho-ethanolamine transferase to the phosphate group of lipid A portion, caused by PhoP-PhoQ and PmrA-PmrB (regulatory system of two components), is also a common mechanism of colistin resistance in Gram-negative pathogens[15-17]. In *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, PhoP/PhoQ causes colistin resistance whereas in *Acinetobacter baumannii*, the colistin resistance mechanism is regulated by the PmrA/PmrB component[18,19].

Since the first report on plasmid-mediated *mcr-1* gene by Lui *et al*. in *E. coli*, incidence studies on the *mcr-1* gene have been performed in *K. pneumoniae, Enterobacter aerogenes*, *Enterobacter cloacae*, *Salmonella species, Cronobacter sakazakaii, Moraxella species, Kluyvera species, Shigella sonnei,* and *Citrobacter* sp.[20-27]. Hitherto, there is no published data regarding plasmid-mediated *mcr-1* gene in MDR *A. baumannii* and *P. aeruginosa*. Therefore, this study was aimed to detect the *mcr-1* gene in these two MDR bacterial species.

METHODS

Sample collection, processing and identification

Between December 2017 and June 2018, 146 non-duplicated clinical isolates of *A. baumannii* (n = 62) and *P. aeruginosa* (n = 84) have been obtained from microbiology laboratories of the four largest tertiary care hospitals, including Hayatabad Medical Complex, North West General Hospital, Rehman Medical Institute, and Combined Military Hospital, in Peshawar, Pakistan. The samples were isolated from various specimens including blood, urine, ulcer swabs, and respiratory secretions; additionally, they were immediately transported in an ice-cold environment to the laboratory of the Institute of Basic Medical Sciences, Khyber Medical University Peshawar, Pakistan for further analysis. Before inoculation, the medium was rendered sterile by incubating the media plates for 24 h at 37 °C. Clear plates with no microbial growth confirmed the sterility of the media. The samples were inoculated on MacConkey agar, Cystein Lactose Electrolyte Deficient (CLED) agar, chocolate agar, and blood agar media. After culturing, plates were incubated for 24 h at 37 °C. All isolates were confirmed as *A. baumannii* and *P. aeruginosa* based on morphological tests (colony morphology and Gram staining results) and biochemical tests (API-10S system, bioMérieux, France). The isolates were stored in the Luria–Bertani broth medium (Oxoid, UK) with 40% glycerol at -80 °C until further analysis.

Antibiotic susceptibility testing

All isolates were subjected to antibiotic susceptibility testing, performed on Muller Hinton agar (MHA) plates by the Kirby–Bauer disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines[28]. Eight antibiotics including Aztreonam (monobactam); amikacin (aminoglycoside); ciprofloxacin and levofloxacin (quinolone); cefepime and cefotaxime (cephalosporin); imipenem (carbapenem); and piperacillin/tozabactam, were used to determine the MDR status of *A. baumannii* and *P. aeruginosa* isolates. The results were compared with the CLSI guidelines.

MIC detection for colistin

Colistin resistance was phenotypically detected by agar dilution and broth micro dilution method, using colistin sulphate powder (Sigma–Aldrich)[29]. The results of MIC were interpreted according to the European Committee on Antimicrobial Susceptibility testing guidelines (EUCAST)[30].

DNA extraction and visualization

For molecular analysis, all phenotypically confirmed colistin-resistant isolates were subjected to the standard alkaline lysis method for plasmid DNA extraction[31]. The extracted DNA was quantified using a micro volume spectrometer (Colibri, Titertek Berthold) and its quality was assessed on 1.5% agarose gel stained with ethidium bromide.

PCR analysis of *mcr-1* gene

The *mcr-1* gene was amplified using gene specific primers (**Table 1**) through conventional PCR, and the cycling conditions used were as follows: denaturation (1 cycle) at 94 ºC for 3 min, denaturation (25 cycles) at 94 ºC for 30 s, annealing at 52 ºC for 30 se, initial elongation at 72 ºC for 1 min, and final extension cycle at 72 ºC for 10 min. The results of the amplified region of the specific gene were visualized on 1.5% agarose gel stained with ethidium bromide.

mcr-1 sequence confirmation and analysis

Sequencing of the PCR products containing amplicons of 309 bp was conducted by Sanger sequencing through an external expertise provider (Macrogen, Korea). The obtained sequences were analyzed using the program "Finch TV" and sequence comparison was performed using the Basic Local Alignment Search Tool available at the NCBI (http://www.ncbi.nlm.nih.gov/blast/).

Nucleotide accession numbers

The nucleotide sequences of the *mcr-1* gene in *A. baumannii* and *P. aeruginosa* have been deposited to GenBank under accession numbers MK340994 and MK340995, respectively.

RESULTS

From 250 clinical specimens collected from four major hospitals in Peshawar, Pakistan in seven months, approximately 146 clinical isolates of *A. baumannii* (n = 62) and *P. aeruginosa* (n = 84) were obtained. The identification was assayed by API-10S strips (**Figure 1**). *A. baumannii* isolates were recovered

TABLE 1: Primers used for PCR amplification of *mcr-1* gene.

Target gene	Nucleotide Sequences 5′ → 3′	Amplicon size	Annealing temperature	Source
mcr-1	MCR1-F: CGGTCAGTCCGTTTGTTC MCR1-R: CTTGGTCGGTCTGTAGGG	309 bp	52 ºC	Liu *et al*.; 2015

from blood, urine, respiratory secretions, and ulcer swabs in percentages of 41.9%, 29.03%, 19.35%, and 9.6% respectively. However, the percentage of *P. aeruginosa* isolates collected from urine, wound, stool, and blood samples were 52%, 23.8%, 2.3%, and 21.4%, respectively.

Antibiotic susceptibility results showed high prevalence of multidrug resistance in *A. baumannii* and *P. aeruginosa* isolates. Approximately 96.7% *A. baumannii* and 83.3% *P. aeruginosa* isolates were detected as MDRs. *A. baumannii* isolates showed the highest resistance for amikacin (90.32%) and aztreonam (83.8%), whereas the lowest resistance rate was observed for cefepime (25.8%) and imipenem (27.4%). Among *P. aeruginosa* isolates, the highest resistance was observed for aztreonam (78.5%) and piperacillin/tozabactam (71.4%) but the lowest resistance was observed for imipenem (39.2%) and cefotaxime (42.8%). The detailed percentages for all tested antibiotics are listed in **Table 2**.

Colistin resistance was found in 9.6% (6/62) of *A. baumannii* and 11.9% (10/84) of *P. aeruginosa* isolates via agar dilution and broth micro dilution method. In the colistin-resistant strains, the MIC values ranged from 8 to 16 µg/ml in *A. baumannii* isolates and 8 to 64 µg/ml in *P. aeruginosa* isolates. The MIC range was determined according to breakpoints suggested in the EUCAST guidelines. Among the 16 colistin-resistant isolates, the *mcr-1* gene was detected in one *A. baumannii* (1.61% of total isolates; 16.6% of colistin-resistant isolates) and one *P. aeruginosa* strain (1.19% of total isolates; 10% of colistin-resistant isolates) (**Figure 2**). However, the remaining 14 colistin-resistant isolates lacked the *mcr-1* gene (**Table 3**).

The nucleotide BLAST results of our study showed 98–99% sequence similarity to the sequences of *mcr-1* present in GenBank.

DISCUSSION

The revival of colistin as the last-line treatment option against infections caused by multiple drug-resistant and extensively drug-resistant Gram-negative bacteria have given some relief to clinicians worldwide. Colistin is used separately or in combination with other antibiotics to effectively treat infections caused by MDR pathogens[32]. Similar to other antibiotics, colistin use is not restricted to humans but has been widely extended to animals for growth promotion and to agriculture for ensuring high yield. This practice has significantly influenced the emergence of colistin-resistant Gram-negative bacteria[33].

In the present study, 16 colistin-resistant isolates have been detected, including 9.6% (6/62) *A. baumannii* and 11.9% (10/84) *P. aeruginosa* isolates, with MIC values ranging from 8 to 16 µg/ml in *A. baumannii* and 8 to 64 µg/ml in *P. aeruginosa* isolates. Most of the *A. baumannii* isolates were recovered from blood specimens (41.9%), which differs from the findings reported by Ishwar *et al*. from India, where most of the *A. baumannii*

FIGURE 1: Biochemical characterization of *A. baumannii* (A) and *P. aeruginosa* (B) by API-10S strips showing a 4-digit code assigned to bacteria based on positive and negative biochemical test results. Code = 2400 confirmed *A. baumannii* whereas code = 2422 confirmed *P. aeruginosa* according to API-10S analytical profile index booklet.

TABLE 2: Antimicrobial susceptibility patterns of *A. baumannii* and *P. aeruginosa* isolates.

Antibiotics	*A. baumannii*		*P. aeruginosa*	
	S%	R%	S%	R%
Aztreonam	16	83.8	21.4	78.5
Amikacin	9.6	90.3	45.2	54.7
Ciprofloxacin	25.8	74.1	29.7	70.2
Levofloxacin	45	54.8	30.9	69
Cefepime	74.1	25.8	35.7	64.2
Cefotaxime	22.5	77.4	57.2	42.8
Imipenem	72.5	27.4	60.7	39.2
Piperacillin/tozabactam	22.5	77.4	28.5	71.4

S: sensitive; **R:** resistant.

TABLE 3: Characteristics of colistin-resistant *A. baumannii* and *P. aeruginosa* isolates.

Colistin resistant strains	Species	Specimen source	Presence of *mcr-1* gene	Colistin MIC (µg/ml)	NCBI accession no
AB015	*A. baumannii*	Blood	-	8	-
AB022	*A. baumannii*	Blood	-	16	-
AB043	*A. baumannii*	Urine	-	8	-
AB047	*A. baumannii*	Blood	+	16	MK340994
AB055	*A. baumannii*	Urine	-	16	-
AB060	*A. baumannii*	Urine	-	16	-
PA02	*P. aeruginosa*	Urine	-	16	-
PA09	*P. aeruginosa*	Urine	+	16	MK340995
PA016	*P. aeruginosa*	Urine	-	16	-
PA033	*P. aeruginosa*	Wound	-	64	-
PA043	*P. aeruginosa*	Stool	-	8	-
PA045	*P. aeruginosa*	Stool	-	8	-
PA066	*P. aeruginosa*	Stool	-	16	-
PA068	*P. aeruginosa*	Blood	-	64	-
PA072	*P. aeruginosa*	Blood	-	64	-
PA074	*P. aeruginosa*	Blood	-	16	-

isolates were recovered from wound swabs (45%)[34]. However, the percentage of colistin-resistant *A. baumannii* isolates in the same study was reported as 7% (7/100), which is in accordance with our study. Oikonomou *et al*. reported 7% (86/1228) colistin-resistant *A. baumannii* isolates with the MIC ranging from 16 to 64 µg/ml[35]. Another study reported 57% (12/21) *A. baumannii* isolates that were resistant to colistin with MIC ranging from 4 to > 128 µg/ml[36].

Colistin-resistant *P. aeruginosa* isolates in our study indicated MICs of 8 to 64 µg/ml, which differ from those reported by Snesrud *et al*.[37]. Lescat *et al*. reported 41.1% (7/17) colistin-resistant *P. aeruginosa* isolates with MIC ranging from 4 to 128 µg/ml[36], in contrast to our observed results regarding colistin-resistance and MIC.

In our study, a single strain of *A. baumannii* and *P. aeruginosa* carrying the *mcr-1* gene was detected. To our

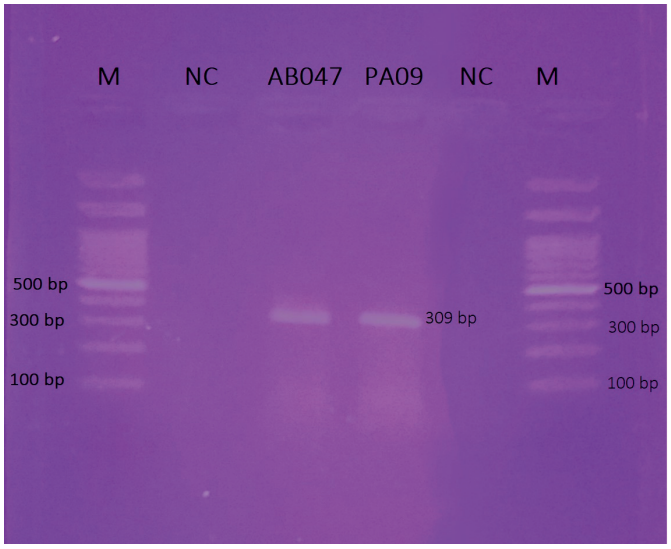

FIGURE 2: PCR amplification of *mcr-1* gene in *A. baumannii* and *P. aeruginosa*: showing 309-bp-amplified fragments of *mcr-1* gene in *A. baumannii* (AB047) and *P. aeruginosa* (PA09). M is the 100-bp DNA marker (Bio-Rad) and NC is negative control.

knowledge, there exists no report on the emergence of *mcr-1* in *A. baumannii* and *P. aeruginosa*. However, several studies have reported the presence of other mechanisms involved in causing colistin resistance in these two critical pathogenic bacteria. Previous studies have reported colistin resistance in *A. baumannii* primarily due to chromosomal mutations, i.e., mechanisms associated with outer membrane changes (mutations in *pmr, lpx, lpsB, lptD, vacJ*) or not associated with outer membrane changes (increase in osmotic tenderness of cell and efflux pumps)[38]. Meanwhile, in *P. aeruginosa*, mutations occurring in two-component regulatory systems are the primary mechanisms attributed to the development of resistance against colistin[18]. In *P. aeruginosa*, the chromosomally encoded *mcr-5* gene has been recently detected but there is no report on its colistin resistance caused by a plasmid-mediated *mcr-1* gene[37].

The present study is the first to report the presence of a plasmid-mediated *mcr-1* gene in *A. baumannii* and *P. aeruginosa* isolated from different clinical specimens in Pakistan. As our study was solely based on the plasmid-mediated detection of the *mcr-1* gene in these two pathogens, we could not investigate further colistin resistance mechanisms in the remaining non-susceptible colistin strains. The findings of our study suggest further experimental procedures to detect plasmid-mediated colistin resistance in these two emerging pathogenic bacteria.

The emergence of plasmid-mediated colistin resistance in *A. baumannii* and *P. aeruginosa* is important owing to the high tendency of the spread of colistin-resistance in clinical settings. It is important to critically analyze and develop guidelines against the use of this last-line treatment drug such that the spread of resistance can be controlled. Development of rapid procedures to detect colistin resistance profiles and implementation of these procedures in hospital laboratories should be encouraged to understand the actual status of global colistin resistance. The use of colistin and carbapenem as a combination therapy may help slow down the process of resistance development and in treating these emerging resistant superbugs.

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Conflict of interest

The authors declare no conflict of interest.

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