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Genomic variations in polymyxin-resistant *Pseudomonas aeruginosa* clinical isolates and their effects on polymyxin resistance

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Abstract

Infection with *P. aeruginosa*, one of the most relevant opportunistic pathogens in hospital-acquired infections, can lead to high mortality due to its low antibiotic susceptibility to limited choices of antibiotics. Polymyxin as last-resort antibiotics is used in the treatment of systemic infections caused by multidrug-resistant *P. aeruginosa* strains, so studying the emergence of polymyxin-resistant was a must. The present study was designed to define genomic differences between paired polymyxin-susceptible and polymyxin-resistant *P. aeruginosa* strains and established polymyxin resistance mechanisms, and common chromosomal mutations that may confer polymyxin resistance were characterized. A total of 116 CRPA clinical isolates from patients were collected from three tertiary care hospitals in China during 2017–2021. Our study found that polymyxin B resistance represented 3.45% of the isolated carbapenem-resistant *P. aeruginosa* (CRPA). No polymyxin-resistant isolates were positive for *mcr* (1–8 and 10) gene and efflux mechanisms. Key genetic variations identified in polymyxin-resistant isolates involved missense mutations in *parR*, *parS*, *pmrB*, *pmrA*, and *phoP*. The *waaL* and *PA5005* substitutions related to LPS synthesis were detected in the highest levels of resistant strain (R1). The missense mutations H398R in ParS (4/4), Y345H in PmrB (4/4), and L71R in PmrA (3/4) were the predominant. Results of the PCR further confirmed that mutation of *pmrA*, *pmrB*, and *phoP* individually or simultaneously did affect the expression level of resistant populations and can directly increase the expression of *arnBCADTEF* operon to contribute to polymyxin resistance. In addition, we reported 3 novel mutations in PA1945 (2129872_A > G, 2130270_A > C, 2130272_T > G) that may confer polymyxin resistance in *P. aeruginosa.* Our findings enriched the spectrum of chromosomal mutations, highlighted the complexity at the molecular level, and multifaceted interplay mechanisms underlying polymyxin resistance in *P. aeruginosa.*

Keywords LPS · Lipid A · Antimicrobial resistance

Introduction

P. aeruginosa is a ubiquitous opportunistic human pathogen that can cause various life-threatening nosocomial infections, affecting immunocompromised people worldwide [1]. In recent years, multidrug-resistant (MDR) and extensively drug-resistant (XDR) *P. aeruginosa* is increasing prevalence and dissemination, with rates of between 15 and 30% in some geographical areas [2]. The infection caused by MDR/XDR-*P. aeruginosa* was very difficult to treat due to its outstanding capacity for being selected and for spreading antimicrobial resistance in vivo [3]. Because of the current shortage of novel antimicrobial drugs to combat infections, polymyxins (colistin and polymyxin B) have been reintroduced into the therapeutic arsenal as last-resort drugs [4]. With the increased use of polymyxins, the emergence of

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polymyxin-resistant *P. aeruginosa* isolates has been reported around the world [5–8].

There are several major mechanisms why there are emergence and dissemination of polymyxin-resistant *P. aeruginosa* strains. First, the development of polymyxin resistance relies on the covalent addition of amino-4-deoxy-L-arabinose (L-Ara4N) and ethanolamine phosphate (PEtN) to the phosphate groups of lipid A [4]. The cellular machinery for covalent modification of negatively charged lipid A of lipopolysaccharide (LPS) with positively charged L-Ara4N 4 is encoded by the *arnBCADTEF-ugd* [9]. Intensive research in the field has demonstrated that the operon is activated by at least five two-component systems (TCS) including PhoP/PhoQ, PmrA/PmrB, ParR/ParS, ColR/ColS, and CprR/CprS [9]. Second, the genetic alterations of proteins PmrB, PhoQ, ParR, and ParS are found in clinical strains exhibiting various degrees of colistin resistance (from 2 mg/L to > 512 mg/L), and all of the reported mutations cause constitutive overexpression of the LPS modification operon *arnBCADTEF-ugd* [4]. Third, the efflux pump MexXY/OprM contributes to the colistin resistance phenotype conferred by mutations activating sensor PmrB, relying on concomitant overexpression of the genes *arnA*, *PA4773-4775*, and *pmrA* [10]. The study highlighted the role played by MexXY/OprM in the adaptation of *P. aeruginosa* to polymyxins. Finally, alterations in outer membrane porins also prevent polymyxin binding to LPS, such as OprH overexpression. OprH is a basic protein that binds to the divalent cation site of LPS, protecting LPS from polymyxin binding [11]. Generally, the understanding of polymyxin resistance mechanisms is limited.

Here, we investigated the genetic differences between polymyxin-resistant and polymyxin-susceptible *P. aeruginosa* clinical isolates using whole genome resequencing. In addition, we explored whether the identified genetic differences are associated with the acquisition of polymyxin resistance in *P. aeruginosa* using molecular techniques. Our results showed that key genetic variations in important two-component systems individually or simultaneously contributed to polymyxin resistance. Secondly, we also found that additional mechanisms, including novel mutation in two-component systems as well as chromosomal mutation and LPS synthesis genes, are involved in the complexity at the molecular level and multifaceted interplay mechanisms underlying polymyxin resistance in *P. aeruginosa.*

Materials and methods

P. aeruginosa isolates and culture

A total of 116 carbapenem-resistant *P. aeruginosa* (CRPA) isolates from various clinical sites between January 2017 and May 2021 were included in this study. All bacterial strains were routinely cultured in Luria–Bertani (LB) medium and stored at − 80 ℃ in the culture medium. Before experiments, strains were cultivated in 20 mL liquid LB medium at 37 ℃ under constant shaking for 18 − 20 h. This study was approved by the First Affiliated Hospital of Kunming Medical University Ethics Research Committee (no. L-20/2021) and exempted the application for informed consent.

Antibiotic susceptibility testing

Identification and susceptibility testing were performed using the VITEK 2 compact automatic bacterial identification system according to Clinical and Laboratory Standards Institute procedures (CLSI M100-S30). Antibiotic susceptibility was determined as minimal inhibitory concentrations (MICs) using the broth dilution method. All susceptibility testing was conducted by the CSLI recommendations. The range of susceptible and resistant polymyxin B concentrations was ≤ 2 and ≥ 4 μg/mL, respectively. The broth microdilution method (BMD) was performed using a cation-adjusted Mueller Hinton II broth (Wenzhou Kangtai Biotechnology Co., Ltd.) following CLSI guidelines. Each test was duplicated, and a third test was performed for discrepant minimal inhibitory concentrations (MICs) or MICs exceeding 1 log2 dilution.

Analysis of LPS by PAGE

LPS was purified from *P. aeruginosa* and was separated on a Bio-Rad Mini-PROTEAN gel apparatus using polyacrylamide gel electrophoresis (PAGE). To visualize LPS on acrylamide gels, the four-step procedure proceeds in sequence using a Silver stain kit (Beyotime Biotechnology), involving a 20-min oxidation step, a 10-min silver staining step, a 10-min color development step, and finally a 5-min color termination step, according to the manufacturer's instructions.

Detection of mcr genes

The genes *mcr1-8* and *mcr-10* were assayed by conventional PCR technique (primer information as shown in Table 1). The reaction conditions were 40 cycles of 95 °C for 1 min, *mcr* genes 52–58 °C for 45 s (the annealing temperature varies according to each primer but is not listed), 72 °C for 1 min, and followed by a final extension of 72 °C for 5 min.

Detection of the efflux pump MexXY/OprM

qRT-PCR was performed to identify the expression level of *mexY*. Sequences are listed in Table 1 according to the related literature [10]; the cycling conditions were as follows: Initial

Table 1 List of related to primers in our study

Gene	Forward primer	Reverse primer
mcr-1	5′-TACAGACCGACCAAGCCGAGAC-3′	5′-AATGACTGCTGAACGCCACCAC-3′
mcr-3	5′-CCTATGACAACACCATCCGCTACAC-3′	5′-ACACGCCATATCAACGCCTTTCTC-3′
mcr-4	5′-GACTATGATCCTCGCCGTGCTAATG-3′	5′-ACTGTATCTTGACTTGGTGCTACTGC-3′
mcr-5	5′-ATGGAGAATGCTGCCCTACTTGTTG-3′	5′-CTGCTGACGATGACGCCTGTTC-3′
mcr-6	5′-ATGTCGTGATGTCGGTATGTTGGTG-3′	5′-ATAGGCGTTGATGAGCGATTGGTG-3′
mcr-7	5′-CGGTTTCGTTATCTCGGTTCCTCTG-3′	5′-GTCGCCTCACTGTTGTTGGTCTC-3′
mcr-8	5′-AACCGCCAGAGCACAGAATTTCC-3′	CGCCATAGCACCTCAACACCTG-3′
mcr-10	5′-TTGCCGCTCTGTATTACCAGGATTATG-3′	5′-TCGCCAATTACCAGGAACATCAGC-3′
mexY	5′-TTACCTCCTCCAGCGGC-3′	5′-GTGAGGCGGGCGTTGTG-3′
pmrA	5′-CACCAGGTGACCCTGTCC	5′-CGTAGAGGCTCTGCTCCAGT-3′
phoP	5′-TCTACCGGGTCAGCGAATAC-3′	5′-GATCAGGATCGGGAAGGACT-3′
parR	5′-AGAATGGTCTGCAGGTGTGC-3′	5′-CGGCTTGATCACGTAGTCGT-3′
cprR	5′-CGCCTGGAAGATCCTTGAGT-3′	5′-CACGTTGAGGGTGTTGCTTT-3′
pmrH	5′-GTTCGTCAGCGACGACAGT-3′	5′-AAACCGGGCTCGATAACTTC-3′
arnA	5′-GTGGCTCGAATACCATGTGA-3′	5′-TGCCGTATTTCACGCAGTAG-3′
PA4773	5′-CAGTGGATCGAGGAAAGCAT-3′	5′-GTACTCCGGCCAGGTATGG-3′
rspL	5′-GTGGTGAAGGTCACAACCTG-3′	5′-CCTGCTTACGGTCTTTGACA-3′
uvrD	5′-CACGCCTCGCCCTACAGCA-3′	5′-GGATCTGGAAGTTCTGCTCAGC-3′

denaturation at 95 °C for the 30 s, 40 cycle denaturation at 95 °C for 5 s, elongation in *mexY* at 55 °C for 25 s, and 1 min at 72 °C. Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method. The *P. aeruginosa* PAO1 was used as control.

Analysis of chromosomal mutation using whole genome resequencing

The genomic DNA from isolates was extracted and purified by using the genomic DNA extraction kit (QIAGEN) for microbial cells. A total amount of 1-μg DNA per sample was used as input material for the DNA library preparation for whole genome resequencing (Novogene Co., Ltd., Beijing, China). Amino acid sequences of 4 polymyxin resistant *P. aeruginosa* (PRPA) isolates were compared with those of 4 polymyxin sensitive *P. aeruginosa* (PSPA) isolates and PSPA reference strain PAO1 (GenBank accession number, AE004091.2). The sequenced data were filtered, and the clean data was used for subsequent analysis. SNP (single nucleotide polymorphism) mainly refers to the DNA sequence polymorphism caused by the single nucleotide variation at the genome level, including transition, and transversion. Indel refers to the insertion and deletion of small fragments in the genome.

Sanger sequencing and qRT-RCR for targeted genes in two-component system and an outer membrane porin gene oprH

Expression levels of *pmrA*, *phoP*, *parR*, *cprR*, *pmrH*, *arnA*, and *PA4773* were determined by qRT-PCR as described previously with some modification [10, 12]. In brief, the total RNA of 4 PRPA and 4 PSPA isolates was extracted from the mid-log phase bacterial culture (optical density at 600 nm of approximately 0.5) using the total RNA extraction kit (Omega, USA) according to the manufacturer's instruction. Reverse transcription reactions were performed in accordance with the protocol for the use of TOYOBO reverse transcriptase ReverTra Ace qPCR RT Master Mix with gDNA remover. Quantification of *pmrA*, *phoP*, *parR*, *cprR*, and *pmrH* transcripts was performed by using SYBR® Green Realtime PCR Master Mix (TOYOBO Co., Ltd., Life Science Department, Osaka, Japan) on quantitative real-time PCR system (SLAN-96P, Shanghai, China). The 30S ribosomal gene *rpsL* was used as a reference gene to analyze gene *mrA*, *phoP*, *parR*, *cprR*, and *pmrH* quantitatively and the expression of the 30S ribosomal gene *uvrD* was assessed in parallel to normalize the transcriptional levels of target genes *arnA* and PA4773. Experiments were repeated with 2 independent cultures, each tested in duplicate (primers as shown in Table 1).

Statistical analysis

Differential gene expression of *pmrA*, *phoP*, *parR*, *cprR*, *pmrH*, *arnA*, *mexY*, and PA4773 between PRPA and PSPA was analyzed with Mann–Whitney *U* test, using a normal significance level $P \leq 0.05$. The SPSS version 25.0 for Windows (SPSS Inc., USA) was used for statistical analysis.

Results

Detection of polymyxin-resistant isolates from carbapenem-resistant *Pseudomonas aeruginosa* isolates

In total, 116 CRPA clinical isolates were determined to be resistant to imipenem or meropenem, and only 4 isolates were determined to be non-susceptible to polymyxin B according to the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (2017) (the clinical resistance breakpoint of polymyxin B against *P. aeruginosa* is ≥ 4.0 μg/mL) (Table 2). The resistance rate of polymyxin B in our study reached 3.45% (4/116, which was relatively low).

Table 2 The source of experimental isolates and the clinical resistance breakpoint of polymyxin B

Bacteria	Source	MIC (μg/mL)
R1	Blood	8
R2	Bronchoalveolar lavage fluid	4
R3	Secretion of wound	4
R4	Sputum	4
S1	Urine	2
S2	Blood	2
S3	Secretion	2
S4	Sputum	2

Detection of lipopolysaccharide (LPS) production

The polymyxins target lipopolysaccharide in the membranes of most Gram-negative species, including *P. aeruginosa*. Considering that changes in the LPS production will enhance the sensitivity of the bacteria to polymyxins, we examined the LPS production in the paired polymyxin-susceptible and polymyxin-resistant *P. aeruginosa* strains and the reference strain PAO1, respectively. As shown in Fig. 1, both resistant and susceptible strains produced observable amounts of LPS, with the strong bands visible between 15 kDa corresponding to the structurally intact lipopolysaccharide. The result demonstrated that LPS production may not be associated with polymyxin resistance in our study.

Detection of mcr plasmid-mediated polymyxin resistance

The plasmid-borne phosphoethanolamine transferases (*mcr*-1 to *mcr*-10) have recently been identified, and these plasmids threaten resistance to polymyxins and increase the rate of dissemination of clinically relevant colistin resistance [13]. The presence or absence of *mcr* genes is determinable by PCR assays, and we examined the *mcr* genes in the paired polymyxin-susceptible and polymyxin-resistant *P. aeruginosa* strains. The results indicate that the *mcr* gene was not found in the two groups of clinical isolates (data not shown).

Detection of the efflux pump MexXY/OprM mediated polymyxin resistance

Previous studies have suggested that MexXY/OprM influences LPS and the expression of and need for LPS

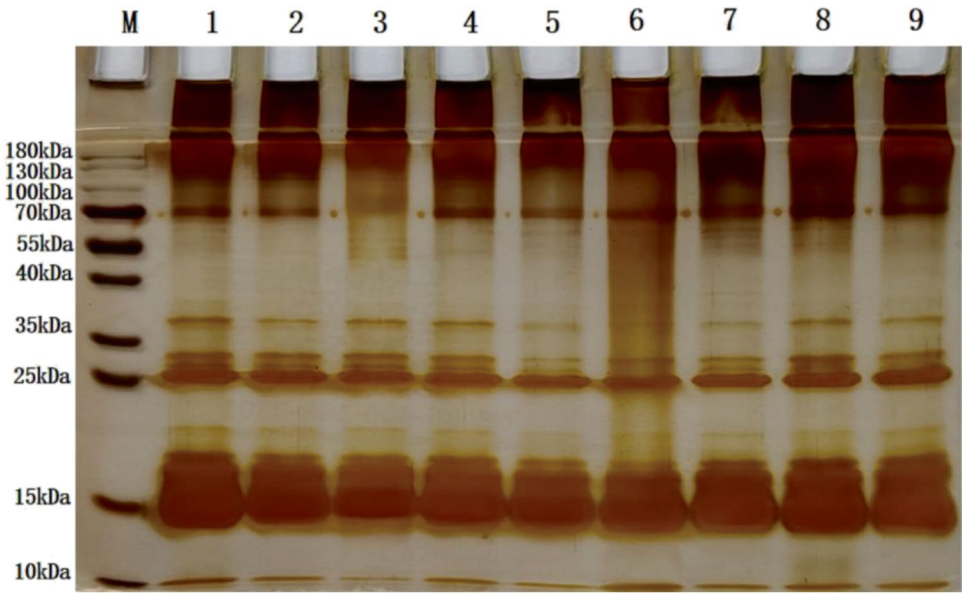

Fig. 1 Silver-stained SDS-PAGE electropherogram of *P. aeruginosa* lipopolysaccharides from polymyxin-resistant *P. aeruginosa* (lane 1–4) to polymyxin-susceptible strains (lane 5–8)

modification loci that promote polymyxin resistance [14]. With further research, *P. aeruginosa* to polymyxin resistance relied on the functional link between the overexpression of the MexXY/OprM pump, the PA4773-PA4774-PA4775 pathway, and Ara4N-based modification of LPS [10]. To assess this, the impact of mexXY and PA4773 expression levels on resistance to polymyxin B was assessed. The results showed that MexXY and PA4773 seem to be uncorrelated with an enhanced susceptibility of *P. aeruginosa* to polymyxin B because the expression levels of mexY and PA4773 in the drug-resistant group were not significantly different from those of PAO1 ($P > 0.05$) (Fig. 2). Even if we found that gene *mexX* and *mexY* mutant is associated with OprM, resistant strains did not show increased sensitive to the polymyxins.

Mutation screening of two-component system regulating LPS modification and an outer membrane porin gene oprH

Polymyxin resistance in *P. aeruginosa* frequently relies on the addition of 4-amino-4-deoxy-l-arabinose (Ara4N) molecules to the lipid A of LPS, through induction of operon *arnBCADTEF-ugd* (*arn*) expression. So far, multiple two-component regulatory systems (PhoP-PhoQ, PmrA-PmrB, CprR-CprS, ColR-ColS, and ParR-ParS) are able to upregulate this operon when *P. aeruginosa* is exposed to polymyxin. We investigated the amino acid alterations of the above two-component regulatory systems, which are known to be associated with colistin resistance in *P. aeruginosa.* We identified amino acid variations in 13 sites, where 5 new mutations were likely associated with colistin resistance because the amino acids in the resistant populations could not be found both in paired sensitive isolates and a wild-type reference strain PAO1 (Table 3). The one missense mutations in *pmrA* (Leu71Arg), four in *pmrB* (Asp70Asn, His340Arg, Tyr345His, Arg390Cys) and two in *parS* (Thr387Ala and His398Arg) were identified in R1-4. Further, (His311Tyr, Glu386Asp, Leu411Met) in *cprS*, (Val55Ile and Ile80Leu) in *parR*, and (Pro31Gln) in *phoP* was identified only in R1, R3, and R4, respectively. Interestingly, gene *oprH* mutation was not detected, which is positively regulated by *phoP*.

To verify whether PmrA-PmrB and PhoP-PhoQ controls the expression of those genes, we performed quantitative real-time PCR assays. Compared to the wild-type reference strain PAO1, mutation of *pmrA*, *pmrB*, and *phoP* individually or simultaneously did affect the expression level of resistant populations (Fig. 3). Strikingly, the expression levels of the *pmrA* gene in the resistant isolates (R1-4), harboring a mutated *pmrB* gene, were found

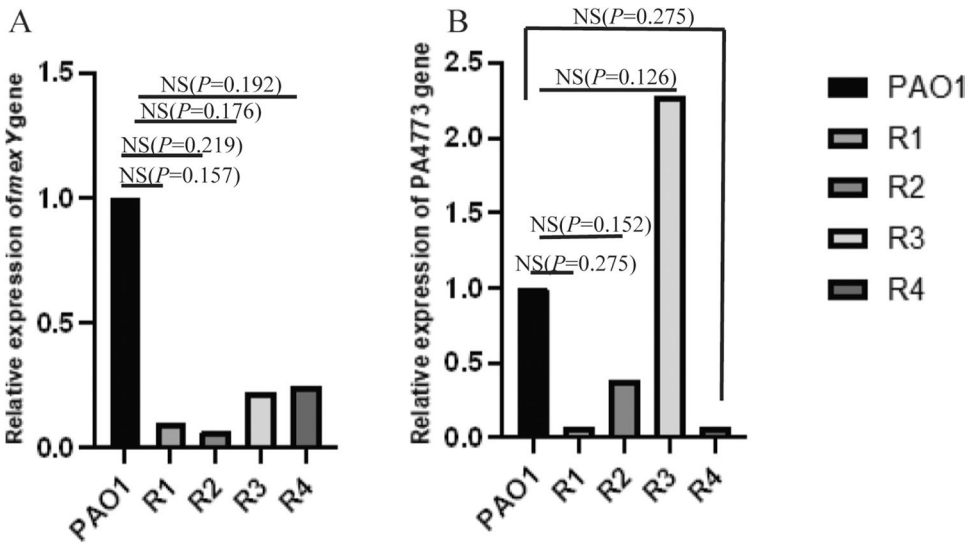

Fig. 2 The relative expression of the genes *mexY* and *PA4773*. The relative expression of the *mexY* **A** and *PA4773* **B** between the reference strain PAO1, and the resistant group was measured by qRT-PCR and normalized to that of the *uvrD* housekeeping gene using the $2.^{-\Delta\Delta Ct}$ method. The experiment was repeated in triplicate. A statistically significant difference between the reference strain PAO1 and resistant group was compared using Mann–Whitney *U* test and represented by $P > 0.05$

Table 3 Amino acid variations in functional domains of Two-component system by sanger-sequencing

Strain	PmrA	PmrB				PhoP	ParR		ParS			CprS	
	71	70	340	345	390	31	55	80	387	398	311	386	411
PAO1	L	D	H	Y	R	P	V	I	T	H	H	E	L
R1	R			H	C				A	R	Y	D	M
R2	R	N	R	H						R			
R3	R			H			I	L		R			
R4				H		Q				R			

to be approximately 9–24-fold higher than that of PAO1. Besides, it has been confirmed that PhoP-PhoQ and other two-component regulatory systems such as PmrA-PmrB, CprR-CprS, ColR-ColS, and ParR-ParS directly control the expression of the *arnBCADTEF* operon [15]. We further confirm the expression of operon *arnA* and *arnB*, and the results showed that the expression level of *arnA* in drug-resistant strains was significantly higher than that of PAO1, while *arnB* only appeared in R1 isolates was significantly higher than that in PAO1 ($P < 0.05$, Fig. 4). In combination, these results suggested that the mutations PmrA-PmrB and PhoP-PhoQ two-component regulatory system can directly increase the expression of *arnBCADTEF* operon to contribute to polymyxin resistance.

Identification of variation in chromosomally encoded genes

Polymyxin resistance in *P. aeruginosa* is known to be principally driven by chromosomal mutations. We analyzed single nucleotide variations by whole genome sequencing to reveal the genetic relationship between the isolate and antimicrobial resistance mechanisms. Our results showed that the types of DNA base mutation included the transition, the transversion, and the deletion, and indels were less frequent than SNPs (Fig. 5A); Besides, common mutations were not found among resistant strains (Fig. 5B). In total, 13 core SNPs (transition or transversion), shared by all 4 isolates, were identified in 11 chromosomal genes (PA0404, PA1153, PA1232, PA1356, PA2069, PA2117, PA2118, PA2852,

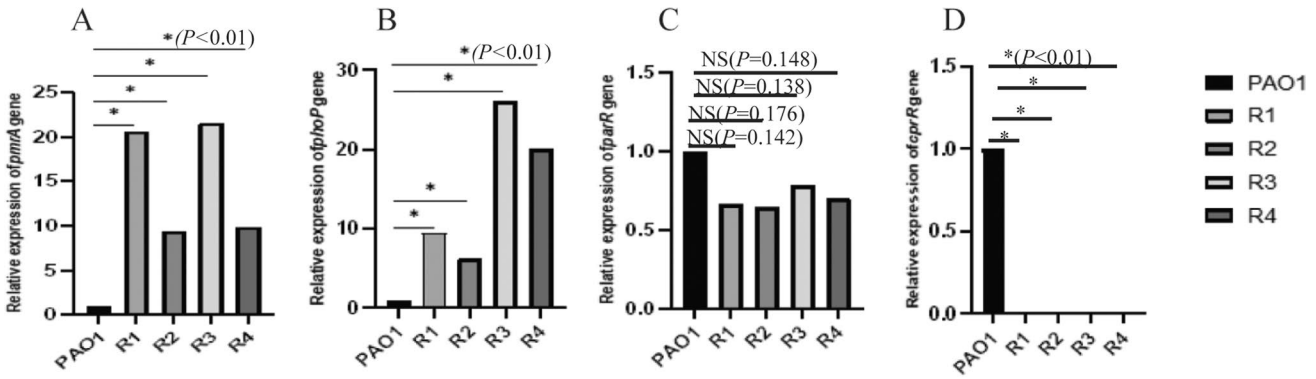

Fig. 3 Relative expression of the genes *pmrA* **A**, *phoP* **B**, *parR* **C**, and *cprR* **D** in the resistant group. The gene *rspL* was taken as the housekeeping gene in for qRT-PCR. A statistically significant difference between the reference strain PAO1, and the resistant group was compared using Mann–Whitney *U* test. $*P < 0.05$ indicated that there is a difference between the strains

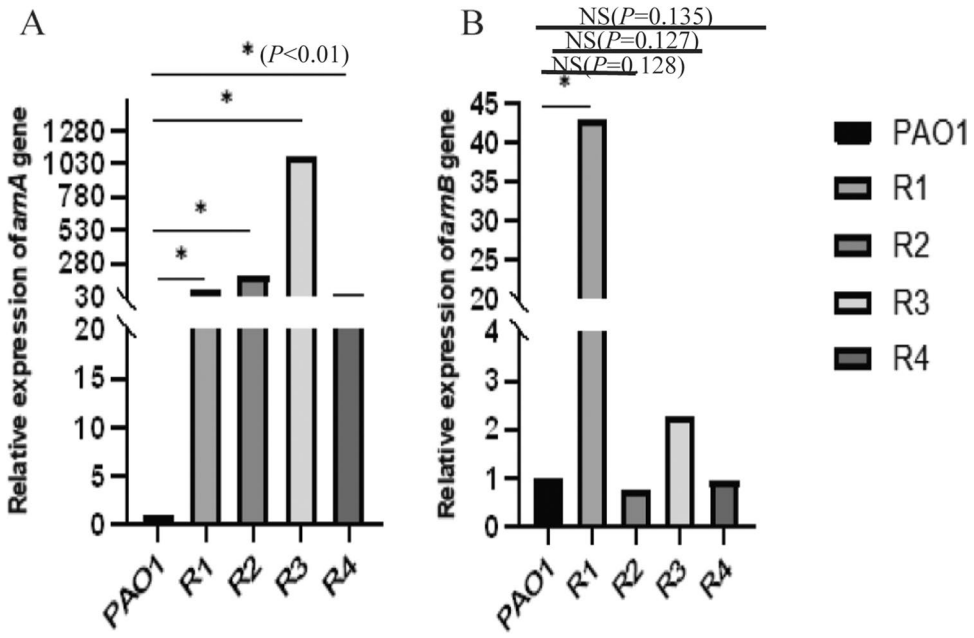

Fig. 4 The relative expression of operon arnA and arnB in the resistant group. **A** The expression of arnA was normalized to uvrD and is reported relative (fold change) to the wild-type *P. aeruginosa* PAO1. **B** The expression of arnB was normalized to rspL. The determination was repeated three times independently. $*P < 0.05$ indicates that there is a difference between the strains

PA3340, PA3414, and PA1945) (Fig. 5C). Although no recognized mutations were observed that easily explain *P. aeruginosa* to polymyxin resistance, three novel nucleotide substitutions within the gene PA1945 region (2129872_A < G; 2130270_A < C; 2130272_T < G) were identified in all 4 isolates, which could affect polymyxin resistance. Furthermore, SNP mutations that have been previously described were also identified between polymyxin-resistant groups or individual isolates, including *mexY*, *mexX*, *PA2928*, *arnA*, *lepA*, *arnT*, *phoP*, *parS*, *parR*, and *eraR* (Table 4). Finally, SNP genetic variations in 65 genes that may impact polymixin resistance were detected among the shared three isolates analyzed in this study (Table 5).

Indels can produce frameshifts in the reading frame of a gene or modify the total number of amino acids in a protein, but they can also affect gene expression levels. Unfortunately, shared indels did not detected in all drug-resistant isolates, while the indels in genes PA0241, PA1297, and PA2065 were shared by three isolates. In particular, a high-impact variant was observed in indels, namely, PA2065, which was shared by three isolates (R2, R3, and R4) and was observed as indel of 39-bp fragments. High-impact variants resulting in protein truncation or triggering loss/gain of function, frameshift variants, or splice donor variants are well known. We cannot rule out the possibility that it may be associated with polymyxin resistance.

Discussion

The hospital environment represents a hotspot for the dissemination of resistant bacterial strains, and *P. aeruginosa* is dominant among the pathogens causing nosocomial infections. Infections caused by *P. aeruginosa* result in serious issues, specifically in the patients of the intensive care unit (ICU) due to the high level of resistance to several antibiotics. Polymyxins as a last-resort antibiotic are used in the treatment of systemic infections caused by multidrug-resistant *P. aeruginosa* strains, so studying the emergence of colistin-resistant was a must. Most published data regarding rates of polymyxin-resistant *P. aeruginosa* are between 0 and 10% with some geographic variance. In the USA, surveillance data from the Sentry database from 2011 to 2017 place colistin resistance at less than 1% [16]. Recent epidemiologic data from ERASnet demonstrated that 4% of overall *P. aeruginosa* isolates were

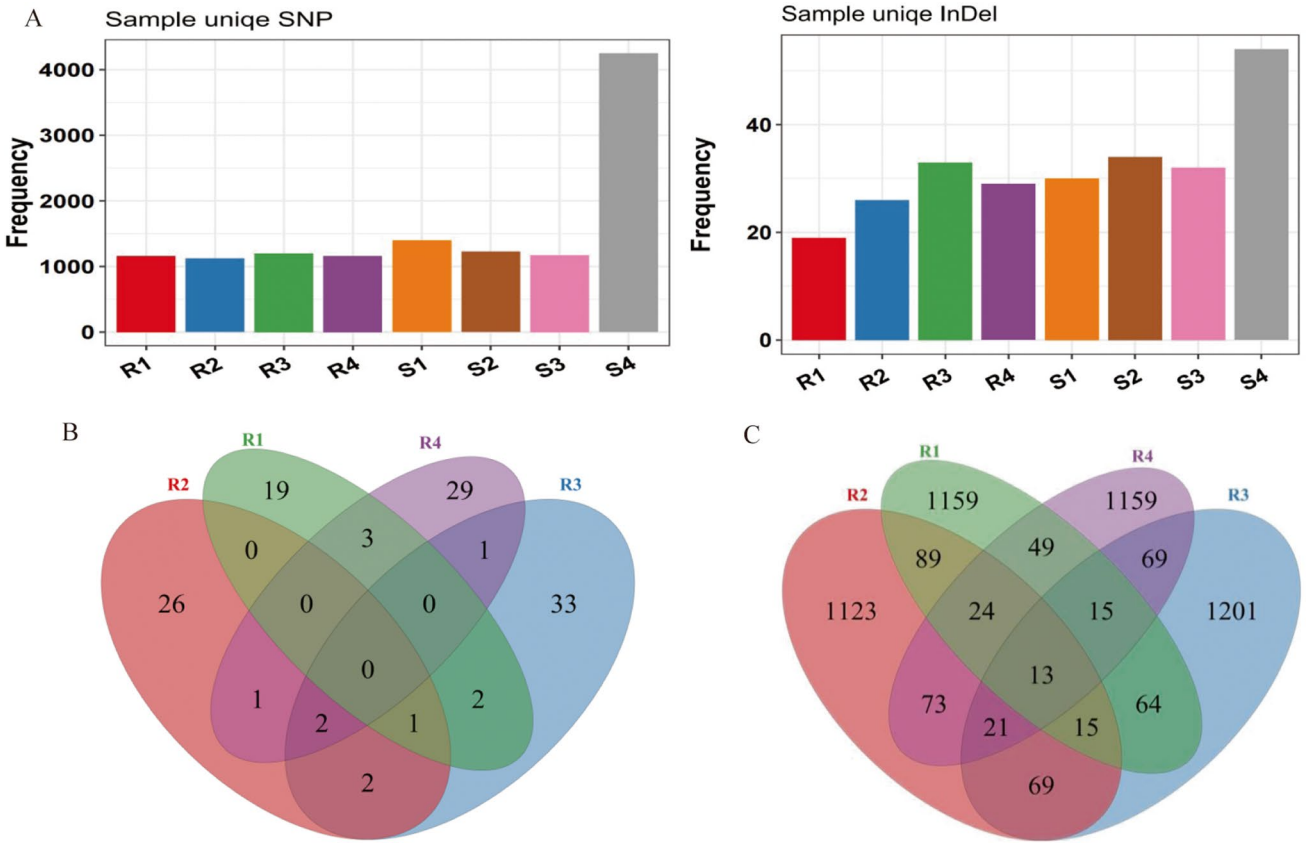

Fig. 5 Genome distribution of SNPs and indels in all strains. **A** The indels were less frequent than SNPs; **B** common mutations were not found among resistant strains; and **C** 13 core SNPs shared by all 4 isolates were identified in 11 chromosomal genes

Table 4 SNP mutations in two-component regulatory system, efflux pump MexXY/OprM and chromosome were identified in our study by whole genome sequencing

Genes	R1	R2	R3	R4
phoP	-	-	-	1277779_C <—> A
parS	1950567_T <—> C	-	-	-
parR	-	-	1952196_T <—> G, 1952271_C <—> T	-
eraR	2165878_G <—> T	2166386_C <—> T	-	-
mexY	2209425_C <—> G, 2210851_C <—> G	-	2208302_C <—> G	2210025_C <—> T
mexX	2211439_C <—> A, 2211462_G <—> T	2212176_C <—> T	2211439_C <—> A	-
PA2928	-	3284489_T <—> G	3283580_C <—> A	3284301_C <—> T
arnA	-	3983564_A <—> G	3982069_G <—> A, 3982657_C <—> T, 3982959_T <—> C	-
arnT	3985520_G <—> T	-	-	3986226_G <—> A, 3986394_G <—> A
lepA	5083349_C <—> T, 5084838_C <—> G, 5086139_G <—> A	-	5082641_T <—> C, 5083742_A <—> G, 5083745_C <—> G, 5083748_A <—> G, 5083750_T <—> C, 5083767_A <—> G, 5083802_A <—> G, 5085160_C <—> A	5083742_A <—> G, 5083745_C <—> G, 5083767_A <—> G, 5084042_G <—> C
prmC	-	-	5231660_G <—> A, 5232318_C <—> G	5231660_G <—> A
speE2	-	5362734_G <—> T	-	-
PA4775	5363217_C <—> A	-	-	-

resistant to colistin in Europe in 2016 [16]. In our study from three tertiary medical hospitals, the prevalence over 2021 was 3.45% (4/116), which is higher than that of the 2.0% average figure for polymyxins reported in 2021 by the China antimicrobial surveillance network (http://www.chinets.com/, accessed on 5 January 2022). The possible reason for this phenomenon is that our study selected carbapenem-resistant *P. aeruginosa* with a high-drug resistance rate, instead of targeting all clinical isolates of *Pseudomonas aeruginosa*. In conclusion, polymyxin B remains a viable treatment option to which the majority of CRPA strains remain susceptible due to its resistance rate being relatively low.

Until now, all reported polymyxin resistance mechanisms are mainly chromosomally mediated, and in rare instances, total loss of the lipopolysaccharide [17] and efflux pump [10]. In our study, both resistant and susceptible strains produced observable amounts of LPS, with the strong bands visible between 15 kDa corresponding to the structurally intact lipopolysaccharide, and resistant strains did not show increased sensitivity to the polymyxins even if we found that genes *mexX* and *mexY* mutant associated with OprM. The result demonstrated that neither LPS production nor the efflux pump MexXY/OprM may be associated with polymyxin resistance in our study.

Significantly, plasmid-mediated colistin resistance mechanisms have been identified worldwide at an alarming rate. All currently known transferable colistin resistance genes included *mcr-1* to *mcr-10* in *Enterobacteriaceae*, and the transfer of *mcr-1* to *P. aeruginosa* in vitro has proven stable [18]. Considering the daily selective pressure in carbapenem-resistant patients receiving colistin by intravenous drip, a transfer of the gene *mcr* to *P. aeruginosa* could be more likely to occur in the population. Results of the PCR screening for *mcr* (1–8 and 10) were negative for all 4 strains, indicating that *mcr* is not the cause of colistin resistance in our study.

Polymyxin resistance to *P. aeruginosa* is always associated with overexpression of the arn operon, which encodes L-Ara4N enzymes in addition to lipid A [19]. The *arn* expression is controlled by a complex regulatory network involving multiple two-component systems (TCSs, such as PmrAB, ParRS, CprRS, and ColRS). Specific mutations within these TCSs triggering constitutive upregulation of the arn operon are typically identified in the high levels of polymyxin-resistant *P. aeruginosa* strains. In this study,

Table 5 Genetic novel variations in 65 genes were detected in our study (list in part)

Genes	R1	R2	R3	R4
phoP	-	-	-	P31Q
parS	T387A	-	-	-
parR	-	-	I80L, V55I	-
eraR	M1I	H171Y	-	-
mexY	D628H, E152D	-	G1002A	D428N
mexX	W358C, R351S	A113T	W358C	-
PA2583	-	E306D	-	-
PA2928	-	D37A	S340I	V100I
arnB	-	-	A20T, A316V	-
arnC	-	-	A39T	-
arnA	-	Q515R	G17S, H213Y, Q315K	-
arnD	-	-	-	F58L, S272N
arnT	A211S	-	-	R446H, R502Q
PA3559	-	R185H	H300L	-
lepA	R303W, T799S,V1233I	-	F67L, S434G,P435A N436D,N442S, N454D H906Q	S434G, P435A N442S,V534L
prmC	-	-	A275V, D56H	A275V
speE2	-	A197S	-	-
PA4775	A7D	-	-	-
pmrB	R390C	D70N, H340R	-	-
waaL	Y58F, A354S, L363M,P364S	-	-	-
PA5005	V313L	-	-	-
PA5548	-	V27I, A313T A367T	-	N2K

the substitutions (i.e., D70N, H340R, and Y345H in PmrB, H398R in parS) that had been reported previously for resistant isolates were detected [20–22]. Therefore, there are some similarities between colistin resistances. In addition, to the best of our knowledge, this is the first report of the R390C substitution in PmrB, T387A substitution in ParS, I80L, V55I substitutions in ParR, and P31Q substitution in PhoP that may mediate polymyxin resistance. Results of the PCR further confirmed that mutation of pmrA, pmrB, and phoP individually or simultaneously did affect the expression level of resistant populations and can directly increase the expression of *arnBCADTEF* operon to contribute to polymyxin resistance. The interaction effect on these genes is also worth investigating.

In fact, the development of high-level polymyxin resistance was also found to be a multistep evolutionary process involving genetic mutations unrelated to L-Ara4N [23, 24]. In this study, *waaL* and PA5005 substitutions related to LPS synthesis were detected in the highest levels of polymyxin-resistant *P. aeruginosa* strains (R1), and four substitutions Tyr58Phe, Ala354Ser, Leu363Met, and Pro364Ser occurred in WaaL, respectively. These results suggested that the mechanism of polymyxin resistance is not only regulated by a two-component system, but also may involve multiple pathways. In addition, we found a new distinctive chromosomal mutation that could correlate with polymyxin resistance to *P. aeruginosa,* located in *PA0404*, *PA1153*, *PA1232*, *PA1356*, *PA2069*, *PA2117*, *PA2118*, *PA2852*, *PA3340*, *PA3414*, and *PA1945*. It was notable that the same 3 SNP loci in PA1945 (2129872_A < G, 2130270_A < C, 2130272_T < G) were detected in the drug resistance group. The role of this gene in polymyxin resistance to *P. aeruginosa* should be further investigated.

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Data availability Some or all data, models, or code generated or used during the study are available from the corresponding author by request.

Declarations

Conflict of Interest The authors declare no competing interests.

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