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Detection of synergistic antimicrobial resistance mechanisms in clinical isolates of *Pseudomonas aeruginosa* from post-operative wound infections

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Abstract

Infections caused by carbapenem-resistant *Pseudomonas aeruginosa* are life-threatening due to its synergistic resistance mechanisms resulting in the ineffectiveness of the used antimicrobials. This study aimed to characterize *P. aeruginosa* isolates for antimicrobial susceptibility, biofilm formation virulence genes, and molecular mechanisms responsible for resistance against various antimicrobials. Out of 700 samples, 91 isolates were confirmed as *P. aeruginosa* which were further classified into 19 non-multidrug-resistant (non-MDR), 7 multidrug-resistant (MDR), 19 extensively drug-resistant (XDR), and 8 pan drug-resistant (PDR) pulsotypes based on standard Kirby Bauer disc diffusion test and pulse field gel electrophoresis. In M9 minimal media, strong biofilms were formed by the XDR and PDR pulsotypes as compared to the non-MDR pulsotypes. The virulence genes, responsible for the worsening of wounds including *LasB*, *plcH*, *toxA*, and *exoU*, were detected among all MDR, XDR, and PDR pulsotypes. Carbapenemase activity was phenotypically detected in 45% pulsotypes and the responsible genes were found as bla_{GES} (100%), bla_{VIM} (58%), bla_{IMP} (4%), and bla_{NDM} (4%). Real-time polymerase chain reaction showed the concomitant use of multiple mechanisms such as *oprD* under-expression, enhanced efflux pump activity, and *ampC* overexpression in the resistant isolates. Polymyxin is found as the only class left with more than 80% susceptibility among the isolates which is an alarming situation suggesting appropriate measures to be taken including alternative therapies.

Key points

- *Multidrug-resistant P. aeruginosa isolates formed stronger biofilms in minimal media.*
- *Only polymyxin antimicrobial was found effective against MDR P. aeruginosa isolates.*
- *Under-expression of oprD and overexpression of ampC were found in resistant isolates.*

Keywords Antimicrobial resistance · Molecular mechanisms · *Pseudomonas aeruginosa* · Carbapenem resistance

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Introduction

P. aeruginosa isolates are the leading cause of wound infections in immunocompromised or critically ill patients at hospitals (Serra et al. 2015). Moreover, due to intrinsic mechanisms for antimicrobial resistance, the treatment is extremely complicated. That is why the limited antimicrobials which were not completely neutralized by some intrinsic mechanisms were designated as antipseudomonal antimicrobials (Giamarellou and Antoniadou 2001). These antimicrobials were majorly from beta-lactams, aminoglycosides, fluoroquinolones, and polymyxins. The beta-lactam antimicrobials further included 4 distinct classes viz., antipseudomonal penicillins (e.g., ticarcillin and piperacillin), antipseudomonal cephalosporins (e.g., cefepime, ceftazidime), monobactams

(aztreonam), and most importantly carbapenems (imipenem, meropenem and doripenem) (Kanj and Sexton 2019).

The antimicrobial resistance against antipseudomonal penicillins or cephalosporins had been extensively reported all around the world in the last century but then the carbapenems were considered the last resort antimicrobials to treat such resistant isolates (Cornaglia et al. 2011; Hancock and Speert 2000). However, studies from the last decade have reported the extensive isolation of carbapenem-resistant *P. aeruginosa* isolates due to the action of carbapenemases which not only neutralized carbapenems but also other beta-lactam antimicrobials including antipseudomonal penicillins and cephalosporins (Ameen et al. 2015; Nordmann et al. 2011). Moreover in developing and third world countries, the prevailing higher resistance rates of pathogens against alternative therapies such as aminoglycosides and fluoroquinolones suggested the evolution of *P. aeruginosa* isolates as superbugs (Nordmann et al. 2007). That is why carbapenem-resistant *P. aeruginosa* have been considered the most dangerous superbugs especially in regions where no policies regarding justified use of antimicrobials have been effectively implemented (Mcdonnell 2018; Tacconelli et al. 2018). The main mechanism responsible for this resistance is the involvement of carbapenemases that actively degrade the β-lactam antimicrobials. However, increased expression of efflux pumps to flush out the carbapenems and decreased expression of oprD porin protein to restrict the entry of carbapenems also synergistically contribute towards higher resistance among the bacteria (Tomás et al. 2010). Furthermore, complexity arose when these bacteria became not only multidrug-resistant but also significantly virulent in addition to switching their motility modes and biofilm formation (Asadpour 2018). Infections caused by such resistant bacteria pose serious challenge to effectively treat the patients.

This study was conducted to identify any of the carbapenem-resistant *P. aeruginosa* isolates from a standard tertiary care hospital setting along with identification of underlying molecular mechanisms and associated factors. This study also focused on the evaluation for the effectiveness of the recommended therapies and the comparison with different regional and international studies.

Methods

Collection of pus samples

The sample collection was performed during Oct 2015 to Oct 2017 from Allied Hospital Faisalabad and District Headquarter Hospital Faisalabad that provide healthcare facilities to more than a million patients every year (Chaudhry 2017). From surgical units of these tertiary healthcare hospitals, 700 pus samples were obtained without any repetition. The majority of the patients were usually discharged after 3 days of surgeries. The patients, who had used post-operative antimicrobials for less than 3 days and/or had completed the prescribed antimicrobial therapies but presented with further deterioration of wounds, were included in this study. The site of infection was washed with sterile normal saline and then the wound was pressed to remove some exudate which was collected with the help of an individually packed sterile cotton swab.

Screening for *P. aeruginosa* isolates

The cultures were directly swabbed on the first half of Nutrient agar plates and MacConkey agar petri plates while the second half of the plates was used to dilute the colonies by streaking at a right angle from the swabbed half (Romo-Ibáñez et al. 2020). The suspected colonies with green pigmentation were then streaked on *Pseudomonas* agar (Oxoid, cat no. CM0559B) plates supplemented with CS supplements (Oxoid, cat no. SR0102E) for phenotypic identification of *Pseudomonas* isolates. Only one colony per pus sample was selected and designated as a separate isolate.

Molecular confirmation of *P. aeruginosa* isolates

The DNA from each isolate was extracted using standard chloroform: isoamyl alcohol extraction protocol (Kumar et al. 2019). The confirmation of these isolates was done by polymerase chain reaction (PCR) using species-specific PCR as described by Spilker and co-workers (Spilker et al. 2004).

Pulse field gel electrophoresis

Pulse field gel electrophoresis is a gold standard method for molecular typing of the multiple isolates of a bacterial species to detect clonal relatedness among the isolates (Neoh et al. 2019). Out of the 91 samples, PFGE of 34 isolates had been completed earlier (Awan et al. 2019). Single colonies from overnight cultures of the remaining 57 isolates were inoculated in 250 µl of LB broth and kept overnight in a shaking incubator at 37 °C and 180 rpm. The cell pellet was obtained which was then washed with normal saline twice to remove media components. The cell suspension was mixed with molten PFGE grade agarose (1.2% in 1 × TBE) and dispensed into the plug molds. The plugs were allowed to cool and then treated with ESP buffer with an overnight incubation at 56 °C. After washing three times with 1 × TE buffer to remove ESP buffer, the plugs were cut into halves. One-half of each plug was then treated with *Xba*1 restriction enzyme for overnight at 37 °C (Alipour et al. 2017). The digested plugs were then washed with 1 × TE buffer and loaded to a 1.2% PFGE grade agarose gel. The gel was then transferred

to the CHEF-DRIII system (Bio-Rad Laboratories, CA, USA) with 0.5 × TBE buffer containing 100 µmol/L thiourea. The equipment was set as angle 120°, voltage 6 V, pulse of 5–50, and duration 22 h. After completing the run, the gel was visualized under gel doc after staining with ethidium bromide (Jordan and Dalmasso 2015). The isolates having 3 or more different bands were considered a different clone.

Phenotypic antimicrobial susceptibility profiling

For antimicrobial susceptibility testing (AST), the Kirby-Bauer disc diffusion method was used as per Clinical Laboratory and Standards Institute (CLSI) guidelines (CLSI 2017). Fifteen antimicrobials representing 7 different antimicrobial groups including penicillins, cephalosporins, monobactams, carbapenems, fluoroquinolones, aminoglycosides, and polymyxins were applied for each isolate on Muller Hinton agar plates. Single colonies were suspended in TSB (Tryptone Soya Broth, Oxoid) tubes and after 16-h incubation at 37 °C, turbidity of the suspensions was adjusted to 0.5 McFarland standard which was prepared indigenously as per previous protocol (Debalke et al. 2018). The diluted suspensions were then spread on Muller Hinton agar plates for bacterial lawn beds. With the use of a disc dispenser (Oxoid, cat no. ST6090), the antimicrobial discs were applied on the swabbed plates. After 16-h incubation at 37 °C, the plates were examined and the zones of inhibitions were measured. For quality control of the used antimicrobials, *P. aeruginosa* ATCC 27,853 was used. The zones were interpreted as per CLSI guidelines and isolates were designated as susceptible, intermediate resistant (sensitive under higher concentration), or resistant accordingly.

For comparison of antimicrobial susceptibility with a comprehensive global report, a "SENTRY Antimicrobial Surveillance Program" report was used that collected and analyzed the antimicrobial susceptibility data of *Pseudomonas aeruginosa* infections from all over the world during 1997–2016 (Shortridge et al. 2019).

Detection of metallo beta-lactamase producers

This test was also based on Kirby Bauer disc diffusion where two different sets of antimicrobials were used. Each set had two discs: first containing a carbapenem antimicrobial (imipenem or meropenem) alone and second having the carbapenem antimicrobial in combination with EDTA (ethylenediaminetetraacetic acid). The EDTA was used as a chelating agent to diminish the activity of any possible metallo beta-lactamase. An isolate that showed a difference of ≥ 7 mm in the resulted zones of inhibitions between an antimicrobial disc and its associated combination disc was designated as metallo beta-lactamase producer (Yong et al. 2002).

Detection of efflux activity

The efflux activity of all the isolates was detected using CCCP (carbonyl cyanide m-chlorophenyl hydrazine) which is an inhibitor of efflux pumps. In this method, CCCP was added as 25 ng/ml in LB broth and dispensed in 96-well plates in alternative rows while the subsequent rows contained only LB broth. The diluted bacterial suspensions (50 µl) were added to each well of the two rows, i.e., with and without CCCP. After 16 h of incubation at 37 °C, the minimum inhibitory concentrations (MICs) were recorded. The isolates showing a more than twofold decrease in MIC were further evaluated by the reverse transcriptase-quantitative PCR (RT-qPCR); however, isolates showing at least fourfold decrease in MIC due to the inhibition by CCCP were considered to have significant efflux activity (Azimi et al. 2016; Filgona et al. 2015).

Motility tests

Two motility tests, swimming and twitching, were performed for each of the *P. aeruginosa* isolates. For the swimming test, TSB was used with 0.3% bacteriological agar in standard plates. Single colonies were picked with an inoculation needle and carefully inoculated on the agar surface at the center of each plate without disturbing the medium. After 16 h of incubation at 37 °C, the zones of propagation were measured in terms of diameter in millimeters (Kilmury and Burrows 2018).

The twitching helps the bacteria to glide through the surfaces. For the twitching test, 1% bacteriological agar was added in TSB and the plates were prepared. After solidification of agar, single colonies were picked with an inoculation needle and stabbed at the center of the plate to the base of the plate. After incubation at 37 °C for 16 h, the agar was carefully removed from the plates. The plate base was stained with 1% crystal violet solution for 5 min and then subjected to a gentle wash under tap water for 5 min. The diameter of the visible zones was measured with the help of a standard scale. *Staphylococcus aureus* was used as negative control being non-motile (Kilmury and Burrows 2018).

Biofilm formation assay using crystal violet method

Two media were used to evaluate the biofilm potential of *P. aeruginosa* isolates in 96-well flat-bottomed polystyrene plates. One enriched media (LB + 0.2% glucose broth) and one minimal media (M9 + 0.2% glucose) were used. The cultures were grown in 1 ml of LB broth at 37 °C and 180 rpm for 16 h to get the optical density OD 600_{nm} as 1. The cultures were then diluted with the two freshly prepared media in ratio 1:100 and distributed as 200 µl per well. The biofilm potential of each isolate in each media was tested in

triplicate. The plates were covered with sealing films and incubated overnight at 37 °C. After incubation, the supernatant from wells was aspirated and washed with 200 µl of sterile 0.9% NaCl (Awan et al. 2019). For staining, 200 µl of 0.1% crystal violet (CV) was added to each well and incubated at room temperature for 10 min. This was followed by twice washing the wells with 200 µl of sterile 0.9% NaCl solution. Then 200 µl of 95% ethanol was added for 10 min to extract the surface-bound crystal violet. Then the solution from these wells was taken in another blank 96-well plate and the optical densities were measured at 570 nm with a Tecan Reader (Awan et al. 2019). For interpretation of data, the "Mean + 3SD" method was used to calculate cutoff values (ODc) to interpret no, weak, moderate, and strong biofilm formation among the tested pulsotypes (Stepanović et al. 2000).

Molecular detection of carbapenemases, aminoglycoside-modifying enzymes and virulence factors

Different virulence genes and genes responsible for enzymatic deactivation of carbapenems and aminoglycosides were targeted using previously reported specific primers by PCR (Table 1) (Elshamy and Aboshanab 2020; Fournier et al. 2013; Gheorghe et al. 2014; Habibi and Honarmand 2015; Poonsuk and Chuanchuen 2012; Sabharwal et al. 2014). For each 25 µl reaction mixture, the final concentrations of reactants were as follows: 1 × Taq buffer, 1.5 mM of $MgCl_2$, 0.2 mM dNTPs mixture, 0.4 µM of each primer, 1 unit *Taq* polymerase with PCR water for volume makeup. DNA template was used as 5 µl from 25 ng/µl stock solution. The thermal cycler program was run as follows: initial denaturation at 95 °C for 5 min, then 30 cycles with denaturation at 95 °C for 30 s, annealing at respective annealing temperature for 30 s and elongation at 72 °C for 30 s. The final extension was performed at 72 °C for 5 min.

Expression analysis of resistance-associated proteins by RT-qPCR

The expression of different resistance-associated proteins was detected in the 40 multidrug-resistant *P. aeruginosa* isolates. The genes targeted for these proteins included *oprD* (for porin), *ampC* (for intrinsic beta-lactamase), while *MexA*, *MexC*, *MexE*, and *MexX* (for efflux pump activity). Total RNA was extracted using the Takara Minibest Universal RNA extraction kit (cat no. 9767, Kusatsu, Japan) as per the manufacturer's instructions. The cDNAs were synthesized by Vazyme HiScript II Reverse Transcriptase (cat no. R223-01, Nanjing, China) according to the manufacturer's instructions. The RT-qPCR was performed using Applied biosystems-StepOnePlus™ Real-Time PCR system. For internal control, the housekeeping *recA* gene was used while *P. aeruginosa* Pao1 strain was used as reference. If the expression of any gene was found twofold increased or decreased, it was considered a significant difference in expression (Chuang et al. 2019).

Statistical analysis

All the data were recorded in Microsoft Excel software 2016. For statistical analysis, one-way ANOVA was used along with pairwise comparison using Tukey's test to find significant differences between resistant and non-resistant pulsotypes in terms of motility testing and biofilm formation. The differences between means were found significant where the *p*-value was less than 0.05.

Results

Identification of *P. aeruginosa* isolates

Out of 700 collected samples, 91 showed characteristic colorless colonies of *P. aeruginosa* with greenish-blue pigmentation on Nutrient, MacConkey, and *Pseudomonas* base (supplemented with CS supplements) agar plates. After DNA extraction of these isolates, all the 91 isolates were confirmed as *P. aeruginosa* by PCR with species-specific amplification of 956-bp fragment of 16S rRNA gene. These isolates were submitted to institutional culture collection as Pak-Pa1 to Pak-Pa91.

Pulse field gel electrophoresis

The 91 isolates when subjected to PFGE analysis showed 53 different PFGE pulsotypes (PT) among which 33 were single isolates while nine and seven pulsotypes consisted of two and three isolates respectively. One pulsotype (PT-11) had five isolates while two pulsotypes (PT-33 and PT-36) contained six isolates each.

Phenotypic characterization of antimicrobial resistance

Out of 53 pulsotypes, eight were PDR (pan drug-resistant: resistant to all tested seven antimicrobial groups) while 19 were XDR (extensively drug-resistant: resistant to five or more antimicrobial groups) including PT-33 and PT-36. Seven pulsotypes were MDR (multidrug-resistant: resistant to three or more antimicrobial groups) while 19 were non-MDR (resistant to less than three antimicrobial groups) that also included PT-11.

Table 1 List of primers used in the study

Sr. #	Resistance genes	Primers sequences (5′-3′)		Amplicon sizes (base pair)
Species-specific identification				
1	*Pa SS*	F	GGGGGATCTTCGGACCTCA	956
		R	TCCTTAGAGTGCCCACCCG	
Carbapenemases				
2	bla_{NDM}	F	GGTTTGGCGATCTGGTTTTC	621
		R	CGGAATGGCTCATCACGATC	
3	bla_{IMP}	F	GAAGGYGTTTATGTTCATAC	587
		R	GTAMGTTTCAAGAGTGATGC	
4	bla_{VIM}	F	GTTTGGTCGCATATCGCAAC	389
		R	AATGCGCAGCACCAGGATAG	
5	bla_{GES}	F	GTTTTGCAATGTGCTCAACG	371
		R	TGCCATAGCAATAGGCGTAG	
6	bla_{KPC}	F	CGTCTAGTTCTGCTGTCTTG	798
		R	CTTGTCATCCTTGTTAGGCG	
7	bla_{SPM}	F	CTGCTTGGATTCATGGGCGC	783
		R	CCTTTTCCGCGACCTTGATC	
8	bla_{SIM}	F	TACAAGGGATTCGGCATCG	570
		R	TAATGGCCTGTTCCCATGTG	
9	bla_{GIM}	F	TCGACACACCTTGGTCTGAA	477
		R	AACTTCCAACTTTGCCATGC	
Aminoglycoside-modifying enzymes				
10	*strA*	F	TGGCAGGAGGAACAGGAGG	405
		R	AGGTCGATCAGACCCGTGC	
11	*strB*	F	GCGGACACCTTTTCCAGCCT	621
		R	TCCGCCATCTGTGCAATGCG	
12	*aph3IIb*	F	GAACGAAACCCAGAGCGACGG	396
		R	CAATCGATGAAGCCGCTGAAGC	
13	*ant2*	F	TGGAGCAGCAACGATGTTACGC	484
		R	CCACTGGTGGTACTTCATCGG	
14	*aadA1*	F	CTCCGCAGTGGATGGCGG	631
		R	GATCTGCGCGCGAGGCCA	
15	*aadA2*	F	CATTGAGCGCCATCTGGAAT	500
		R	ACATTTCGCTCATCGCCGGC	
16	*aadB1*	F	CTAGCTGCGGCAGATGAGC	300
		R	CTCAGCCGCCTCTGGGCA	
17	*aadB*	F	GAGCGAAATCTGCCGCTCTGG	320
		R	CTGTTACAACGGACTGGCCGC	
Virulence genes				
18	*pilB*	F	ATGAACGACAGCATCCAACT	826
		R	GGGTGTTGACGCGAAAGTCGAT	
19	*lasB*	F	GGAATGAACGAGGCGTTCTC	300
		R	GGTCCAGTAGTAGCGGTTGG	
20	*plcH*	F	GAAGCCATGGGCTACTTCAA	307
		R	AGAGTGACGAGGAGCGGTAG	
21	*exoU*	F	GGGAATACTTTCCGGGAAGTT	428
		R	CGATCTCGCTGCTAATGTGTT	
22	*toxA*	F	GGAGCGCAACTATCCCACT	150
		R	TGGTAGCCGACGAACACATA	
23	*fliC*	F	GGCAGCTGGTTNGCCTG	Type-a = 1.02 kb Type-b = 1.25 kb
		R	GGCCTGCAGATCNCCAA	

The highest antimicrobial resistance (> 70% pulsotypes) was observed against single ingredient penicillins that was reduced (42% pulsotypes) when tested in combination with a beta-lactamase inhibitor. More than 40% of pulsotypes showed resistance against monobactam, cephalosporins, and carbapenems. Similarly, more than 50% of pulsotypes showed resistance against fluoroquinolones and aminoglycosides. The least resistance (19% of pulsotypes) was observed against polymyxin that was under the acceptable limit as shown in Table 2.

The comparison of antimicrobial susceptibility data with the "SENTRY Antimicrobial Surveillance Program" report is given in Table 3. It was observed that the overall antimicrobial resistance of our isolates was much higher than the overall resistance for individual drugs mentioned in the SENTRY's report. Moreover, it was also found during the comparison that the aminoglycoside resistance in our XDR pulsotypes was much higher than that of XDR isolates in the global study.

All 24 carbapenem-resistant pulsotypes (out of 53) showed the production of carbapenemases, by metallo beta-lactamase assay, which is responsible for the inactivation of not only carbapenems but also penicillins and cephalosporins. For efflux pump inactivation, only 3 (5.6%) pulsotypes showed a fourfold decrease in MICs when tested in synergy with CCCP (carbonyl cyanide m-chlorophenyl hydrazine) confirming the involvement of efflux pumps; however, 23 (43%) pulsotypes showed a twofold decrease in MICs.

Molecular characterization

The carbapenemase production was detected phenotypically in all 24 carbapenem-resistant pulsotypes including PT-33 and PT-36. Using PCR, bla_{GES} was detected in all 24 (100%) carbapenemase-producing pulsotypes, and bla_{VIM} was detected in 14 (58%) pulsotypes while bla_{NDM} and bla_{IMP} were detected in one pulsotype each. According to RT-qPCR of these carbapenem-resistant pulsotypes, significant activities of Mex-EF and Mex-AB efflux pump were found in 14 (58%) and 8 (33.3%) pulsotypes respectively. In addition to that, significant over-expression of *ampC* gene and under-expression of *oprD* gene were observed in 9 (37.5%) and 6 (25%) carbapenem-resistant pulsotypes respectively. Out of 24 carbapenem-resistant pulsotypes, 2 MDR, 7 XDR, and 4 PDR pulsotypes showed concomitant involvement of a carbapenemase enzyme and an efflux pump. In addition to that, 6 XDR and 2 PDR pulsotypes showed concomitant use of carbapenemase enzyme and efflux pump with either over-expression of *ampC* or under-expression of *oprD* while only 3 PDR isolates showed all four mechanisms simultaneously.

In the case of 32 aminoglycoside-resistant pulsotypes, the most prevalent genes were *strA* and *strB* (14/32, 43.75%) which were followed by *ant2* (13/32, 40.62%) and *aadB* (9/32, 28.1%). The genes *aadA1* and *aadA2* were detected only in 2 and 1 pulsotypes respectively. Significant overexpression of Mex-XY efflux pump was found in 20/32 pulsotypes by RT-qPCR. However, in 8/32 aminoglycoside-resistant pulsotypes, no tested molecular resistance mechanism was detected.

Table 2 Antimicrobial susceptibility testing results for 53 pulsotypes

Antimicrobials	Non-MDR (19)			MDR (7)			XDR (19)			Overall (53)		
	%R	%I	%S	%R	%I	%S	%R	%I	%S	%R	%I	%S
Aztreonam	**16.0**	58.0	26.0	57.0	29.0	14.0	84.0	16.0	0.0	59.0	30.0	11.0
Imipenem	**0.0**	11.0	89.0	43.0	0.0	57.0	74.0	0.0	26.0	47.0	4.0	49.0
Meropenem	**0.0**	0.0	100.0	43.0	0.0	57.0	74.0	0.0	26.0	47.0	0.0	53.0
Doripenem	**0.0**	0.0	100.0	29.0	14.0	57.0	74.0	0.0	26.0	45.0	2.0	53.0
Cefepime	**0.0**	21.0	79.0	43.0	28.5	28.5	89.0	0.0	11.0	53.0	11.0	36.0
Ceftazidime	**11.0**	5.0	84.0	43.0	14.0	43.0	74.0	16.0	11.0	51.0	9.0	40.0
Ciprofloxacin	**5.0**	0.0	95.0	57.0	0.0	43.0	100.0	0.0	0.0	60.0	0.0	40.0
Levofloxacin	**5.3**	5.3	89.4	57.0	0.0	43.0	89.0	11.0	0.0	56.6	5.6	37.8
Amikacin	**0**	0	100	57.0	0.0	43.0	95.0	0.0	5.0	57.0	0.0	43.0
Gentamicin	**0**	5	95	57.0	0.0	43.0	89.4	5.3	5.3	55.0	3.5	41.5
Tobramycin	**0**	0	100	57.0	0.0	43.0	84.0	5.0	11.0	53.0	2.0	45.0
Ticarcillin	42	42	16	100.0	0.0	0.0	100.0	0.0	0.0	79.0	15.0	6.0
Piperacillin/tazabactam	**5.3**	26.3	68.4	**14.0**	43.0	43.0	63.0	26.0	11.0	41.5	24.5	34.0
Piperacillin	100	0	0	71.0	29.0	0.0	53.0	47.0	0.0	72.0	28.0	0.0
Polymyxin B	**0**	0	100	**0.0**	0.0	100.0	**11.0**	0.0	89.0	**19.0**	0.0	81.0

Bold numbers indicate an effective antimicrobial (%R≤20) against a set of pulsotypes

R resistant pulsotypes, *I* intermediate pulsotypes/sensitive under high concentration, *S* sensitive pulsotypes, *MDR* multi-drug-resistant pulsotypes, *XDR* extensively drug-resistant pulsotypes, and *Non-MDR* non multi-drug-resistant pulsotype

Table 3 Comparison of Antimicrobial susceptibility data with a SENTRY global study

Antimicrobials	This study (% R)	SENTRY report (2013–2016 data)					(Percentage antimicrobial resistance)			
		All regions (% R)	North America (% R)	Europe (% R)	Asia–Pacific (% R)	Latin America (% R)	This study MDR data (% R)	SENTRY Report (1997–2016) MDR data (% R)	This study XDR data (% R)	SENTRY Report (1997–2016) XDR data (% R)
Meropenem	47.2	22.6	18.2	29.4	17.2	32.3	42.9	59.1	73.7	70.0
Cefepime	52.8	16.9	13.9	21.8	13.2	22.8	42.9	36.7	89.5	46.3
Ceftazidime	50.9	19.2	14.9	25.6	18.1	25.4	42.9	58.8	73.7	68.2
Ciprofloxacin	60.4	24.3	22.0	29.1	16.4	28.9	57.1	67.9	100.0	79.2
Amikacin	56.6	6.9	3.3	12.2	5.0	13.2	57.1	26.4	94.7	33.8
Tobramycin	52.8	11.2	7.0	17.7	6.3	19.4	57.1	51.5	84.2	62.9
Piperacillin/tazabactam	41.5	22.6	18.4	29.2	19.8	26.9	14.3	51.4	63.2	59.8

R resistant pulsotypes/isolates as per CLSI guidelines, *SENTRY Report* "SENTRY Antimicrobial Surveillance Program" report

Out of 53 pulsotypes, 38 were flagellar type-a while 15 were flagellar type-b. The virulence genes *LasB*, *PlcH*, and *toxA* were found in all pulsotypes while *exoU* gene was detected significantly higher (p-value = 0.038) among resistant (MDR, XDR, and PDR) pulsotypes than non-MDR pulsotypes. The PCR-based detection of different virulence genes among 53 pulsotypes is given in Table 4.

Motility testing

The minimum zone for both swimming and twitching motility assay was 2 ± 0.7 mm, the maximum zone for swimming assay was 50 ± 2.82 mm, and the maximum zone for twitching assay was 23.5 ± 1.4 mm. The overall results for both assays are depicted in Fig. 1. There was no significant difference (p-value = 0.116) among twitching patterns of resistant and non-resistant pulsotypes. However, the swimming motility of the non-MDR pulsotypes was found significantly less (p-value < 0.0001) than the resistant pulsotypes especially when compared with XDR pulsotypes (p-value = 0.004) and PDR pulsotypes (p-value = 0.0003).

Biofilm formation assay

Out of 53 pulsotypes, 15 pulsotypes formed strong biofilms in both enriched and minimal media as shown in Table 5. In case of LB broth, no significant difference (p-value = 0.44) was observed between biofilm formation of non-MDR and MDR pulsotypes. However, in M9 minimal media, a significant difference (p-value = 0.000172) between biofilm formation of non-MDR pulsotypes and MDR pulsotypes was found. It was further observed that XDR (p-value = 0.0048) and PDR (p-value = 0.037) pulsotypes formed better biofilms than non-MDR pulsotypes.

Discussion

The carbapenem-resistant *P. aeruginosa* isolates have been nominated as the most critical pathogens which urgently require newer strategies or therapies (Shrivastava et al. 2018). In the absence of antimicrobial stewardship programs and systemic collection of antimicrobial susceptibility profiling data, the individual reports by various research organizations are somewhat indicative of the prevailing resistance patterns in a region (Nathwani et al. 2019; NHSRC 2017; Saleem et al. 2018; Vernet et al. 2014; WHO 2015). International guidelines for antimicrobial use recommend an alternative antimicrobial when a recommended antimicrobial showed resistance in more than 20% of bacterial isolates in a community (Gupta et al. 2011).

The international guidelines consider antipseudomonal beta-lactams as imperative antimicrobials for the

Table 4 Occurrence of virulence genes among 53 pulsotypes

Virulence genes	Function	Presence in pulsotypes
pilB	Type 4 fimbrial biogenesis protein PilB (Mitov et al. 2010)	26
LasB	Degrade mucins and surfactant proteins (Alcorn and Wright 2004)	53
plcH	Distinctive vasculitis and poor wound healing (Vasil et al. 2009)	53
exoU	Cytotoxic necrotizing toxin with phospholipase activity (Gendrin et al. 2012)	31
toxA	Inhibiting protein synthesis and leading to cell death (Ertugrul et al. 2018)	53
fliC	Flagellar type a (Ertugrul et al. 2018)	38
	Flagellar type b (Ertugrul et al. 2018)	15

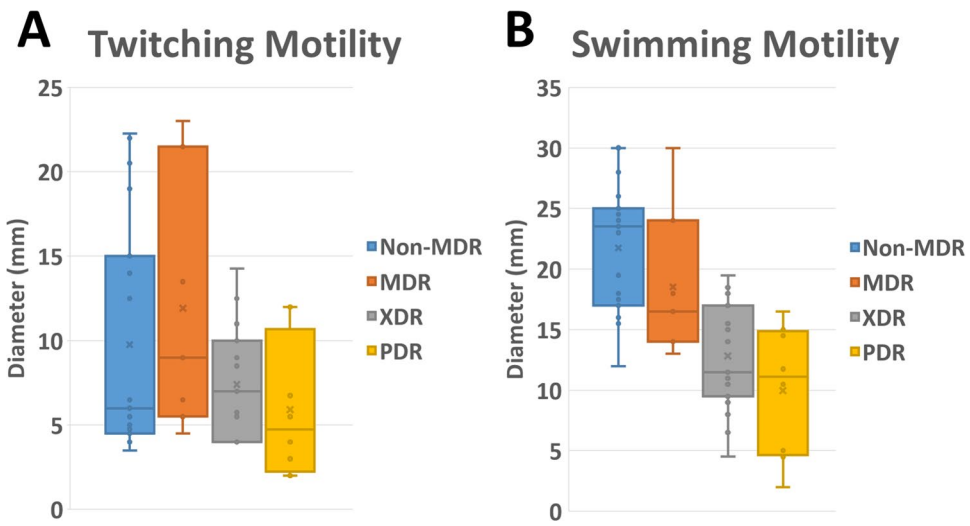

Fig. 1 Box plots for motility assays among the non-MDR, MDR, XDR, and PDR pulsotypes. **A** Twitching motility assay. **B** Swimming motility assay

Table 5 Potential of Biofilm formation among 53 pulsotypes

Minimal medium M9 minimal media + 0.2% glucose				
Enriched medium LB	Biofilm formation	Strong	Moderate	Weak
	Strong	15	18	5
	Moderate	4	5	1
	Weak	3	2	0

management of pseudomonal infections (El Zowalaty et al. 2015). The European and US surveillance studies have shown the effectiveness of anti-pseudomonals against majority of *P. aeruginosa* isolates but still multidrug-resistant isolates have also been reported with lower susceptibilities (Lob et al. 2019; Pfaller et al. 2017; Sader et al. 2017; Shortridge et al. 2019). In this study, anti-pseudomonal penicllins, cephalosporins, and monobactam were less effective (effective against ≤ 40% isolates) as observed in a similar reports (Ullah et al. 2016). The most effective of these β-lactam antimicrobials were carbapenems with resistance among 40% of the *P. aeruginosa* isolates, well above the breakpoint but in line with the recent studies conducted in tertiary healthcare hospitals in Islamabad, Rawalpindi, and Peshawar cities of Pakistan (Saleem et al. 2018). However, a recent report from Karachi found imipenem effective only in 19% of the *P. aeruginosa* isolates which is the highest reported resistance from Pakistan against any carbapenem (Farooq et al. 2019; Ullah et al. 2016). These reports have been published in last 2 years which showed that this increase in carbapenem resistance has evolved recently as multiple studies had reported good to moderate effectiveness of carbapenems in Pakistan just 5 years back (Mansoor et al. 2016; Shah et al. 2015).

Among the tested alternative anti-pseudomonals, fluoroquinolones and aminoglycosides showed effectiveness against less than 40% isolates which is similar to the studies from Peshawar, Islamabad, and Karachi (Ullah et al. 2016). Only a report from Islamabad showed ciprofloxacin as completely effective against *P. aeruginosa* isolates (Saleem et al. 2018). The genes coding for aminoglycoside-modifying enzymes were found with higher frequencies as compared to a previous report (Braun et al. 2018), particularly *strA*, *strB*, and *ant2* genes that were detected in more than 65% isolates. Such higher frequencies of these genes show the resistance potential of the MDR isolates. However, in our

study, the polymyxins were the sole effective drugs against the isolates which is also supported by the study in Karachi. However, a recent report from Karachi, Pakistan, showed that colistin might not remain an effective drug for long as it reported 57% effectiveness in *P. aeruginosa* isolates (Saleem and Bokhari 2019).

This increase in carbapenem resistance might be due to the recent shift towards this class by the physicians as other antimicrobials have badly failed to treat any serious infections which might be due to synergistic use of multiple resistance mechanisms. The most important of all β-lactamases are carbapenemases which not only denature carbapenems but also the rest of the β-lactam antimicrobials (Sacha et al. 2008). All 24 carbapenem-resistant pulsotypes showed involvement of a carbapenemase which were majorly encoded by bla_{GES} (100%) and bla_{VIM} (58%) genes while bla_{IMP} and bla_{NDM} were found in only two isolates. In Pakistan, carbapenemase-encoding genes bla_{IMP} and bla_{NDM} have been previously reported (Akhtar et al. 2018; Saleem et al. 2018; Sepehri et al. 2014) with similar detection rates, but in our study, the detection rate for bla_{VIM} gene (58%) was relatively higher. Similarly, efflux pump activity along with decreased *oprD* expression and *ampC* overexpression was also found by RT-qPCR in this study which has not been previously reported from this region. We have also found synergistic use of different mechanisms that resulted in much higher resistance levels among these isolates.

The resistance patterns of the isolates were quite alarming as the percentage of multidrug resistance was found 64% (34 pulsotypes) among the 53 pulsotypes. Many studies from different countries have reported PDR and XDR among various Gram-negative bacterial isolates (Falagas and Bliziotis 2007; Karakonstantis et al. 2019) including *P. aeruginosa* with their potential to transfer and spread the resistance genes (Freschi et al. 2018). We have found 8 (15%) PDR isolates, 19 (36%) XDR, and 7 (13%) MDR isolates while only 19 (36%) isolates were non-MDR as resistant to less than 3 antipseudomonal classes (Fig. 1). This is in line with the results of a study from Peshawar, Pakistan, which had reported 10% isolates as PDR (Samad et al. 2017); however, this study has not tested any representative antimicrobial from polymyxins which might have an impact on the resistance data. The PDR isolates reported earlier from Pakistan were 4–5% while the majority of the isolates were reported as MDR (Gill et al. 2011; Ullah et al. 2016). We have also compared the antimicrobial resistance data of our MDR and PDR pulsotypes with that of "SENTRY Antimicrobial Surveillance Program" report to identify the similarities and differences. It was observed that the overall resistance in our *P. aeruginosa* pulsotypes was much higher than that of isolates collected globally during 2013–2016 (Shortridge et al. 2019). This might be due to higher proportion of non-MDR isolates among the collected isolates as more than 70% *P. aeruginosa* isolates were non-MDR. Contrary to that, when the MDR and XDR data was compared, the differences were found much lower. Still, the aminoglycoside resistance in our MDR and XDR isolates was much higher than acceptable limits (< 20%) in comparison to the global data which signifies the role of local antimicrobial stewardship program in predicting best available treatment options.

There was no significant difference found between biofilm formation among MDR and non-MDR pulsotypes in LB medium; however, it was found that XDR and PDR isolates formed better biofilms than non-MDR pulsotypes in M9 minimal medium. The association of biofilm formation with antimicrobial resistance has been established by our previous study (Awan et al. 2019); however, certain studies have reported no association between these two parameters. This may be due to the variation in the chemical composition of the media tested (Cepas et al. 2019). However, this area requires further investigation with a bigger sample size and multiple control factors for a conclusive inference.

Another interesting association of motility with multidrug resistance was found in our study as resistant (XDR and PDR) showed relatively less swimming potential than the sensitive pulsotypes (Table S1). The increase in drug resistance associated with the non-motile behavior of bacterial pathogens has already been reported (Rodulfo et al. 2019). Some studies have suggested that the virulence of such non-motile isolates is lower than motile bacteria due to reduced ability to attach the cellular surfaces(Zolfaghar et al. 2003). However, some studies have also suggested that this might be an escape strategy by *P. aeruginosa* isolates to host macrophages as these are less likely to be found by host defense system (Amiel et al. 2010). Meanwhile, it is also observed by some researchers that the reduction in twitching ability had reduced the cytotoxic potential of isolates (Zolfaghar et al. 2003).

We conclude that the polymyxins are now the last antimicrobials that still have effectiveness against MDR *P. aeruginosa* isolates as the carbapenems, although they showed effectiveness against more than 50% of the isolates, but it is still not enough to be recommended against pseudomonal infections. Moreover, the presence of PDR isolates, expressing multiple resistance mechanisms along with strong biofilm formation potential and ability to escape the immune system, in hospital settings, is a serious threat for the indigenous healthcare system. The most effective strategy is the use of synergistic antimicrobials which may depend upon the pharmacokinetic and pharmacodynamics properties of the antimicrobials under pathological conditions.

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Author contribution A. B. A. designed, performed, and analyzed experiments and wrote the paper; A. Y. designed and supervised the expressional studies; P. S. supervised the biofilm and PFGE studies and provided guidance in statistical analysis and improving the manuscript; Y. S. supervised the phenotypic characterization methods; A. A. designed and supervised the whole project and finalized the manuscript.

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Data availability All materials are available by the corresponding author.

Declarations

Ethics approval The study was approved by the Institutional Medical Ethics Committee of National Institute for Biotechnology and Genetic Engineering College, Pakistan Institute of Engineering and Applied Sciences (NIBGE-C, PIEAS) Faisalabad, Pakistan (ID no. 14092015).

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