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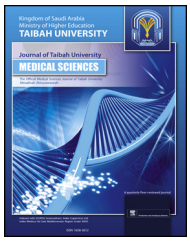

Original Article

Phenotypic and genotypic characterization of clinical *Pseudomonas aeruginosa*

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الملخص

أهداف البحث: الزائفة الزنجارية، هو ممرض انتهازي يمكن أن يسبب العديد من التهابات المستشفيات. يساهم تكوين الأغشية الحيوية ومقاومة الأدوية والحركة في ضراوة الزائفة الزنجارية. يدرس هذا البحث الحد الأدنى من تركيز المثبط للكوليستين، وتكوين الأغشية الحيوية، ووجود جينات (مود أ) و (بسل أ) وأنواع الحركات في مقاومة الأدوية المتعددة والحساسية للأدوية المتعددة للزائفة الزنجارية.

طريقة البحث: تمت دراسة 62 من الزائفة الزنجارية من القيح و 18 من عينات البول لقابليتها للمضادات الحيوية الشائعة الاستخدام، وتركيز الكوليستين المثبط الأدنى عن طريق تخفيف أجار والقدرة على تشكيل الأغشية الحيوية بواسطة طريقة لوحة عيار مكروي. تم اختبار جميع عزلات الزائفة الزنجارية المقاومة للأدوية المتعددة والحساسية للأدوية المتعددة لوجود جينات (مود أ) و (بسل أ) بواسطة مقايسة التفاعل السلسلي للبوليميراز وأنواع مختلفة من الحركات باستخدام وسائط معينة.

النتائج: من بين 40 عزلة مقاومة للأدوية المتعددة و 40 عزلة متعددة الحساسية للأدوية ، كانت 17 عزلة مقاومة للكوليستين و 23 عزلة مقاومة متوسطة للكوليستين، على التوالي. أظهرت تسعة عزلات صديد مقاومة للأدوية وثلاث عزلات بول مقاومة للأدوية جميع الأنواع الثلاثة من القدرة على الحركة. أظهرت 13 عزلة صديد حساسة للأدوية و 4 عزلات بول متعددة الحساسية للأدوية كلا من حركة السباحة والتجمع. لم تظهر العزلات الحساسة للأدوية المتعددة حركية الوخز. كان هناك عدد أكبر من السلالات المقاومة للأدوية المتعددة منتجة قوية للأغشية الحيوية (عدد = 19) بينما كان عدد أكبر من السلالات المعرضة للأدوية المتعددة (عدد = 24) منتجة معتدلة للأغشية الحيوية. كانت سبعة وعشرون عزلة مقاومة للأدوية المتعددة و 28 عزلة حساسة للأدوية المتعددة إيجابية لكل من جينات (مود أ) و (بسل أ). من بين العزلات القوية المكونة للقيح الحيوي، عدد أكبر من العزلات المعرضة للأدوية المتعددة (عدد = 13) تحتوي على جينات (مود أ) و (بسل أ) عند مقارنتها بالعزلات متعددة الحساسية للأدوية.

الاستنتاجات: تظهر النتائج التي توصلنا إليها بوضوح وجود علاقة ذات دلالة إحصائية بين تكوين الأغشية الحيوية القوية وجينات (مود أ) و (بسل أ) ومقاومة الأدوية في الزائفة الزنجارية المعزولة من العينات السريرية. من المستحسن إجراء دراسات إضافية لاستكشاف الجينات والعوامل الأخرى المسؤولة عن تكوين الأغشية الحيوية الضعيفة والمتوسطة ومقاومة الأدوية.

الكلمات المفتاحية: النمط الظاهري؛ النمط الجيني؛ فلم حيوي؛ جين مود أ؛ جين بسل أ؛ الزائفة الزنجارية

Abstract

Objectives: *Pseudomonas aeruginosa* is an opportunistic pathogen that can cause many nosocomial infections. Biofilm formation, drug resistance, and motility contribute to virulence in *P. aeruginosa*. This study assessed the colistin minimum inhibitory concentration (MIC), biofilm formation, presence of *mod A* and *psl A* genes, and types of motilities in multidrug-resistant (MDR) and multidrug-susceptible (MDS) *P. aeruginosa*.

Methods: Sixty-two *P. aeruginosa* from pus and 18 from urine samples were studied for their susceptibility to commonly used antibiotics, colistin MIC by agar dilution, and biofilm-forming ability by the microtiter plate method. All MDR and MDS *P. aeruginosa* isolates were tested for the presence of *mod A* and *psl A* genes by PCR, and different types of motilities using specific media.

Results: Among the 40 MDR and 40 MDS isolates, 17 each were colistin-resistant and 23 each were colistin-

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intermediate. Nine MDR pus isolates and three MDR urine isolates showed all three types of motilities. Thirteen MDS pus isolates and four MDS urine isolates showed both swimming and swarming motility. MDS isolates did not show twitching motility. A higher number of MDR strains were strong biofilm producers (n = 19), whereas a higher number of MDS strains (n = 24) were moderate biofilm producers (p = 0.023). Twenty-seven MDR and twenty-eight MDS isolates were positive for both m*od A* and *psl*A genes. Among the strong biofilm-forming pus isolates, a greater number of MDR isolates (n = 13 each) had *modA* and *psl*A genes compared to MDS isolates (*mod*A p = 0.017; *psl*A p = 0.014).

Conclusions: Our findings clearly showed a statistically significant association among strong biofilm formation, *mod*A, *psl*A genes, and drug resistance in *P. aeruginosa* isolated from clinical samples. Additional studies are needed to explore other genes and factors responsible for weak and moderate biofilm formation and drug resistance.

Keywords: Biofilm; Genotype; *mod A* gene; Phenotype; *Pseudomonas aeruginosa*; *psl A* gene

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes surgical site infections, urinary tract infections, nosocomial infections, septicemia, lower respiratory tract infections, and infections in immunocompromised individuals.[1] This is a prototype organism for studying bacterial virulence and social traits as it causes mortality and morbidity in patients with cystic fibrosis.[2,3]

Infections associated with multidrug-resistant (MDR) *P. aeruginosa* strains are difficult to manage, as mucoid and non-mucoid strains show variation in their resistance patterns.[4] MDR strains of *P. aeruginosa* produce enzymes such as extended-spectrum β-lactamases and metallo-β-lactamases, which inactivate β-lactam drugs that in turn account for treatment failures.[5] MDR and extensively drug-resistant (XDR) strains are dangerous as they can spread resistance to other bacterial species in the hospitals and persist on hospital devices.[6] MDR strains exhibit resistance to a minimum of one agent in three or more antimicrobial categories. XDR strains stay susceptible to only one or two categories of antibiotics.[7] Colistin is often the only effective antibiotic against MDR *P. aeruginosa* infections. Colistin resistance in *P. aeruginosa* is due to chromosomal mutations, either modification of lipid A or loss of lipopolysaccharide.[8]

P. aeruginosa exhibit three types of motilities: swimming, swarming mediated by flagella, and twitching by type 4 pili. Motility mediated by flagella help the bacterium move away from deleterious environment, and pili helps with attaching to the surfaces, thereby contributing to the virulence. Switching to a sessile lifestyle is a survival advantage and indicator of lower virulence.[9]

Biofilm formation is a survival advantage and *P. aeruginosa* first becomes sessile, adhere to surfaces, form microcolonies, and get fixed in extracellular polymeric substances to form a biofilm.[10] Loss of motility contributes to biofilm formation, which helps the bacteria protect itself from the effects of antibiotics. Thus, by forming biofilm, *P. aeruginosa* can persist against the effects of phagocytosis, oxidative and nutritional stresses, antibiotics, and metabolic waste.[11] Biofilm formation helps the organism survive on medical devices and enhances drug resistance in *P. aeruginosa*, thereby contributing to chronic infections in patients on mechanical ventilators, with burn wounds and cystic fibrosis.[12,13]

Three exopolysaccharides alginate synthesis (alg8 gene), *P. aeruginosa* exopolysaccharide coded by polysaccharide synthesis locus *psl*, (*pslA* gene), and pellicle operon Pel (*pel* A to G) play roles in biofilm formation. Among these, p*slA* plays an essential role in the initial steps of biofilm formation.[14] Dafopoulou et al.[15] showed that *ppK* and *modA* are needed for biofilm formation, and loss of these genes leads to defective biofilm production in *Acinetobacter baumanii*. However, Azmi et al.[16] concluded that the colistin-resistant (CR) strains of *P. aeruginosa* lack *ppK* and *modA* genes and do not form a biofilm. Thus, when bacteria develop colistin resistance, they lose the *modA* gene and fail to form biofilm. Because *psl*A and *mod*A genes take part in biofilm formation in *P. aeruginosa,* we studied the biofilm-forming ability and associated genes (*psl*A and *mod*A), along with different types of motilities in MDR and multidrug-susceptible (MDS) *P. aeruginosa* strains.

Materials and Methods

Isolation and identification of P. aeruginosa

P. aeruginosa (n = 80) isolated from pus and urine samples in the Department of Microbiology, Kasturba Medical College (KMC) Hospital (Mangalore, Karnataka, India), from January 2021 to June 2021, were included in the study. Chemicals, media, and antibiotic discs used for this study were purchased from Hi-Media Laboratories Pvt Ltd. (Mumbai, India). Pus and urine samples were cultured on sheep blood agar and MacConkey's agar. Oxidase positive and non-lactose-fermenting colonies on MacConkey's agar were identified by biochemical reactions using the VITEK-2 system (bioMerieux Inc., Durham County, NC, USA).

Antimicrobial susceptibility testing

The antibiotic susceptibility pattern of *P. aeruginosa* was studied with the Kirby Bauer disk diffusion method. The clinical isolate and control strain of *P. aeruginosa* ATCC 27853 were inoculated onto Muller Hinton agar (MHA) after adjusting the turbidity to 0.5 McFarland standard. Gentamicin (10 μg), amikacin (30 μg), ceftazidime (30 μg), cefoperazone (75 μg), imipenem (10 μg), and meropenem (10 μg) discs were placed on the inoculated MHA agar plates and incubated for 24 h at 37 °C. Results

were interpreted as susceptible, intermediate, or resistant using Clinical and Laboratory Standards Institute (CLSI) guidelines.[17]

Minimum inhibitory concentration determination by the agar dilution method

The agar dilution method was performed following CLSI guidelines for the detection of colistin minimum inhibitory concentration (MIC).[17] Three to five colonies of *P. aeruginosa* were mixed in 4–5 mL sterile saline to prepare the inoculum. The turbidity of the inoculum was adjusted to 0.5 McFarland standard using saline. Then 10 μL diluted (1:10) inoculum were placed on a colistin agar plate using a loop and incubated for 16–20 h at 35 °C. The MHA plate with and without colistin, inoculated with *P. aeruginosa* strain ATCC 27853, was used as a growth control. The MHA agar plate with the lowest concentration of colistin that inhibited the growth of test organisms completely was considered the MIC as per CLSI 2020 guidelines.[17] An MIC ≤2 μg is interpreted as intermediate and an MIC ≥4 μg as CR.[17]

Detection of motility

Swimming motility

P. aeruginosa isolates were subcultured onto Luria–Bertani (LB) agar. Overnight cultures of *P. aeruginosa* on LB agar were picked using a toothpick and stabbed in swim plates (tryptone broth with 0.3% agarose). The plates were incubated at 37 °C for 12–14 h.[18]

Swarming motility

P. aeruginosa isolates to be tested were subcultured on LB agar. Colonies taken from an overnight swim plate were inoculated on swarm plates. Swarm plates consist of nutrient broth supplemented with 0.5% of glucose and agar.[18]

Twitching motility

Overnight culture of *P. aeruginosa* grown on LB agar was stabbed to the bottom of twitch plates (LB agar plates) using a toothpick, and plates were incubated at 37 °C for 24 h. The zone of motility in agar was measured at the end of the incubation period. A diffuse interstitial zone indicated twitching motility.[18]

Detection of biofilm by the microtiter plate method

The test strain was inoculated in 1 mL trypticase soy broth (TSB) containing 1% glucose. After incubation at 37 °C for 4 h, the turbidity of culture was adjusted to 0.5 McFarland standard by diluting in saline. Then 200 μL turbidity-adjusted culture was added in triplicate to the wells of microtiter plates (Labtech Medico Pvt Ltd., Kerala, India). *Enterococcus faecalis* ATCC 29212 and sterile TSB were used as positive and negative controls, respectively. Microtiter plates were incubated for 72 h at 37 °C aerobically. The contents of the well were discarded and washed with phosphate-buffered saline. Methanol was added to the wells and incubated for 20 min to fix the biofilm after which it was air-dried. Crystal violet solution (0.1%) was used for staining the biofilm. Wells were washed and dried. Dye was resolubilized using 200 μL of 80% ethanol for each well.[19] Absorbance was measured at 550 nm in a microtiter plate reader (Multiskan™ FC Filter-based Microplate Photometer; Thermo Fisher Scientific, Waltham, MA, USA). Mean optical density (OD) of the isolates (ODi) was compared with the mean OD of the negative control (ODc). The ratio of the optical densities, $\gamma = \frac{ODi}{ODc}$ was used to classify the isolates' ability to form biofilm as follows[20]:

- *No biofilm* if $\gamma \leq 1$
- *Weak biofilm* if $1 < \gamma \leq 2$
- *Moderate biofilm* if $2 < \gamma \leq 4$
- *Strong biofilm* if $\gamma > 4$

Detection of pslA and modA genes by PCR

PCR was performed on all 80 clinical *P. aeruginosa* isolates. The boiling method was used for DNA extraction from bacterial isolates. Five colonies of each *P. aeruginosa* were suspended in 100 μL PCR grade water, heated for 15 min in a dry bath at 100 °C, and centrifuged for 5 min at 14,000 rpm; 2 μL supernatant was used as DNA for PCR. *pslA* and *mod A* genes were amplified by PCR using specific primers as shown in Table 1. *P. aeruginosa* ATCC 27853 was used as a positive control.[16] Master mix with nuclease-free water was used as a negative control. The PCR reaction mixture consisted of 5 μL template DNA, 2.5 μL 10X Taq polymerase buffer, 0.25 μL each of primers, 0.25 μL dNTPs, 0.2 μL Taq DNA polymerase, and 16.55 μL nuclease-free water to a final volume of 25 μL[16]. The amplicons were separated on a 1.5% agarose gel at 120 V for 45 min and stained with ethidium bromide for 15 min. Gels were visualized under a ultraviolet transilluminator, and gel photographs were captured and bands were analyzed.[21]

Statistical analyses

The result were analyzed using SPSS version 20.0 (Statistical Package for the Social Sciences; IBM Co., NY, USA). All experiments were performed three times, and data are presented as the mean ± standard deviation, percentages, and proportions. The chi-square test was utilized for comparing the proportions. $P < 0.05$ was considered statistically significant.

Abbreviations: CR, Colistin-resistant; MDS, Multidrug-susceptible; MDR, Multidrug-resistant.

Results

P. aeruginosa *isolates included in the study*

In this study, a total of 80 *P. aeruginosa* were included, which consisted of 62 isolates from pus and 18 from urine samples.

Antibiotic susceptibility of P. aeruginosa *isolates*

Based on antimicrobial susceptibility testing by the disc diffusion method, the isolates were categorized as MDR and MDS. Among the 80 isolates tested, 40 were resistant to gentamicin (10 μg), amikacin (30 μg), ceftazidime (30 μg),

Table 1: Primer sequences used for this study.

Primers	Primer sequence	Amplicon size	PCR conditions			Reference
			Denaturation	Annealing	Extension	
*psl*A (F) *psl*A (R)	5′-TGGGTCTTCAAGTTCCGCTC- 3′ 5′-ATGCTGGTCTTGCGGATGAA-3′	119 bp	Initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s	52 °C for 40 s	72 °C for 50 s and final extension at 72 °C for 10 min	(21)
modA (F) *modA* (R)	5′-CTTCCTGCTCCAGTTTCG- 3′ 5′- GCCAAGGAATTCGAGAAA- 3′	130 bp	Initial denaturation at 93 °C for 5 min followed by 30 cycles of denaturation at 93 °C for 1 min	57 °C for 1 min	72 °C for 1 min followed by a final extension at 72 °C for 10 min	(16)

cefoperazone (75 µg), meropenem (10 µg) and were categorized as MDR. Since these 40 isolates were resistant to antibiotics belonging to the class aminoglycosides, cephalosporins, and carbapenems, they were categorized as MDR. Other 40 isolates susceptible to all of the tested antibiotics were categorized as MDS. Among the MDR isolates, 32 were from pus and 8 were from urine. Of the MDS isolates, 30 were from pus samples and 10 were from urine samples.

Determination of colistin MIC by the agar dilution method

All 40 MDR and 40 MDS *P. aeruginosa* isolates were assessed for colistin MIC by the agar dilution method. Among the MDR and MDS, *P. aeruginosa* isolates 17 each were CR and 23 each were colistin-intermediate (CI) respectively. There were no colistin-susceptible isolates.

Motility

Swimming and swarming motilities facilitated by flagella and twitching motility by type 4 pili were studied. Nine MDR pus isolates (5 CR & 4 CI) and three MDR urine isolates (1 CR & 2 CI) showed all three types of motilities. Thirteen MDS pus isolates (7 CR & 6 CI) and four MDS urine isolates (2 each of CR & CI) showed both swimming and swarming motility. The type of motility showed by

Table 2: Comparison of types of motilities among multidrug-resistant and multidrug-sensitive *Pseudomonas aeruginosa* isolates from pus and urine samples.

Sample	Type of motility	Multidrug-resistant (MDR) strains		Multidrug-sensitive (MDS) strains	
		Colistin- resistant (CR) strains	Colistin-intermediate (CI) strains	Colistin- resistant (CR) strains	Colistin-intermediate (CI) strains
Pus	**Swimming**	9 (42.9%)	12 (57.1%)	11 (50.0%)	11 (50.0%)
	Swarming	12 (57.1%)	9 (42.9%)	9 (60.0%)	6 (40.0%)
	Twitching	7 (58.3%)	5 (41.7%)	0	0
	Total	**28**	**26**	**20**	**17**
Urine	**Swimming**	3 (60.0%)	2 (40.0%)	2 (28.6%)	5 (71.4%)
	Swarming	1 (33.3%)	2 (66.7%)	2 (33.3%)	4 (66.7%)
	Twitching	1 (33.3%)	2 (66.7%)	0	0
	Total	**5**	**6**	**4**	**9**

Total and sub totals are in bold.

Table 3: Biofilm forming ability of multidrug-resistant and multidrug-sensitive *Pseudomonas aeruginosa* isolates from pus and urine samples.

Sample	Type of biofilm	Multidrug-resistant (MDR) strains		Multidrug-sensitive (MDS) strains		Total
		Colistin-resistant (CR) strains	Colistin-intermediate (CI) strains	Colistin-resistant (CR) strains	Colistin-intermediate (CI) strains	
Pus	**Weak**	2 (40.0%)	3 (60.0%)	2 (33.3%)	4 (66.7%)	11 (17.7%)
	Moderate	4 (36.4%)	7 (63.6%)	10 (50.0%)	10 (50.0%)	31 (50.0%)
	Strong	7 (43.8%)	9 (56.3%)	2 (50.0%)	2 (50.0%)	20 (32.3%)
	Sub total	**13**	**19**	**14**	**16**	**62 (100.0%)**
Urine	**Weak**	0	0	1 (100.0%)	0	01 (5.6%)
	Moderate	4 (80.0%)	1 (20.0%)	0	4 (100.0%)	09 (50.0%)
	Strong	0	3 (100.0%)	2 (40.0%)	3 (60.0%)	08 (44.4%)
	Subtotal	**4**	**4**	**3**	**7**	**18 (100.0%)**

Total and sub totals are in bold.

Table 4: Distribution of *ModA* and *Psl A* genes in *Pseudomonas aeruginosa* isolated from pus and urine samples.

Sample	Gene	Result	Multidrug-resistant (MDR) strains		Multidrug-sensitive (MDS) strains	
			Colistin- resistant (CR) strains	Colistin -intermediate (CI) strains	Colistin- resistant (CR) strains	Colistin -intermediate (CI) strains
Pus	***Mod A***	Positive	9 (36.0%)	16 (64.0%)	11 (64.0%)	15 (57.7%)
		Negative	4 (57.1%)	3 (42.9%)	3 (75.0%)	1 (25.0%)
		Total	**13**	**19**	**14**	**16**
	Psl A	Positive	8 (34.8%)	15 (65.2%)	11 (45.8%)	13 (54.2%)
		Negative	5 (55.6%)	4 (44.4%)	3 (50.0%)	3 (50.0%)
		Total	**13**	**19**	**14**	**16**
Urine	***Mod A***	Positive	3 (50.0%)	3 (50.0%)	2 (25.0%)	6 (75.0%)
		Negative	1 (50.0%)	1 (50.0%)	1 (50.0%)	1 (50.0%)
		Total	**4**	**4**	**3**	**7**
	Psl A	Positive	2 (40.0%)	3 (60.0%)	2 (22.2%)	7 (77.8%)
		Negative	2 (66.7%)	1 (33.3%)	1 (100%)	0
		Total	**4**	**4**	**3**	**7**

Total and sub totals are in bold.

MDR and MDS *P. aeruginosa* clinical isolates from pus and urine are shown in Table 2. No statistically significant association was seen between types of motilities shown by MDR and MDS *P. aeruginosa* isolated from pus and urine samples. However, a statistically significant difference was seen in the twitching motility of MDR & MDS strains ($p = 0.029$).

Detection of biofilm production by the microtiter plate method

Biofilm formation that attaches to the polystyrene walls of the microtiter plate was done for all of the isolates. All 80 isolates of *P. aeruginosa* were able to form a biofilm. Isolates were grouped as weak, moderate, and strong biofilm formers as shown in Table 3. A higher number of MDR strains ($n = 19$) were strong biofilm producers, whereas a higher number of MDS strains ($n = 24$) were moderate biofilm producers. ($p = 0.023$). However, a statistically significant difference was not seen in the biofilm-forming ability of pus and urine isolates. Both MDR and MDS strains with and without twitching motilities formed biofilm.

Detection of modA and pslA genes by PCR

Uniplex PCR was done to detect *ModA* and *PslA* genes in MDR and MDS *P. aeruginosa* clinical isolates. The distribution of *modA* and *PslA* genes among MDR and MDS *P. aeruginosa* isolates is shown in Table 4 and Figure 1. Twenty-seven MDR isolates were positive for both genes,

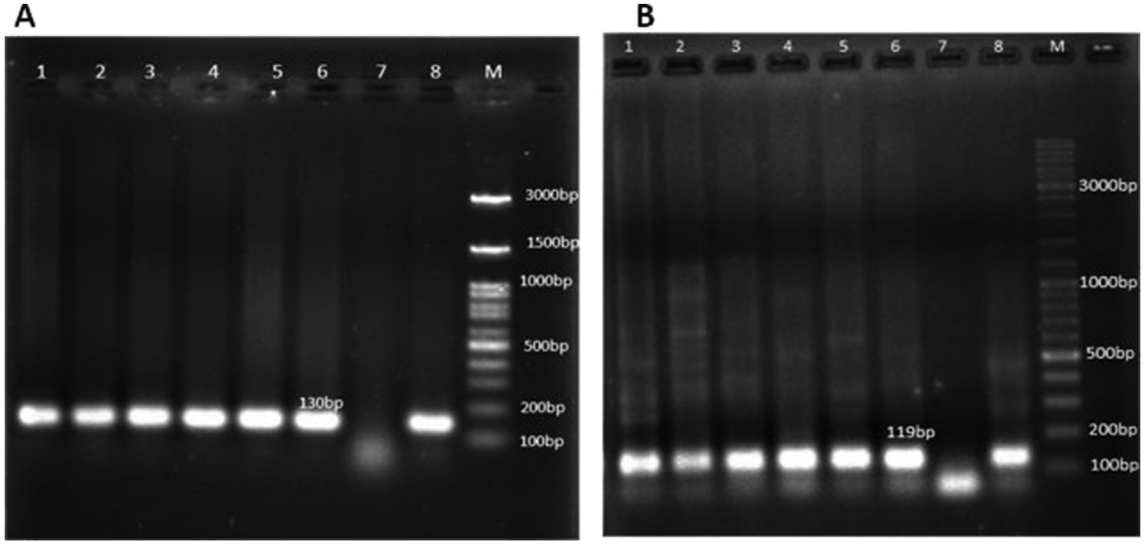

Figure 1: A: Agarose gel image of uniplex PCR for detection of the *Mod A* gene among *P. aeruginosa* isolates. Lanes: M-100 bp DNA ladder, **7**: Negative control (NC) - Master mix with nuclease-free water, **8**: Positive control (PC) - *P. aeruginosa* ATCC 27853, **1–6** isolates positive for *ModA* genes. **B: Agarose gel image of uniplex PCR for detection of the *Psl A* gene among *P. aeruginosa* isolates. Lanes: M**-100 bp DNA ladder, **7**: Negative control (NC) - Master mix with nuclease-free water, **8**: Positive control (PC) - *P. aeruginosa* ATCC 27853, **1–6** isolates positive for the *Psl A* gene.

which included eight CR, fourteen CI isolates from pus, and two CR and three CI isolates from urine. However, 28 MDS isolates were positive for both genes, which included eight CR, twelve CI isolates from pus, and two CR and six CI isolates from urine. A statistically significant difference was not found in the presence of *modA* and *plsA* genes among the MDR and MDS isolates from different samples (pus isolates: *Mod A* $p = 0.379$, *Psl A* $p = 0.455$; urine isolates: *Mod A* $p = 0.80$, *Psl A* $p = 0.163$; $p < 0.05$). Among the strong biofilm-forming pus isolates, a greater number of MDR isolates ($n = 13$ each) had *Mod A* and *psl*A genes compared to the four MDS isolates (*Mod*A $p = 0.017$; *psl*A $p = 0.014$). Thus, our findings clearly showed a statistically significant association among strong biofilm, the presence of the *modA* gene, and MDR among the pus isolates.

Discussion

In the current study, 80 *P. aeruginosa* isolates were characterized for their antibiogram, swarming, swimming and twitching motilities, biofilm-forming capacity, and the presence of associated genes namely *psl*A and *mod*A. The 32 isolates from pus and 8 from urine were MDR, as they were resistant to most of the antibiotics tested. Since they were resistant to more than three antibiotics of different classes, they were categorized as MDR.[7] The rest of the 30 isolates from pus and 10 from urine were susceptible to all of the tested antibiotics and were grouped as MDS. A recent Indian study reported that 34% of *P. aeruginosa* is MDR.[22] The resistance rate seen in the present study is 50%. This difference in the resistance rate could be due to the difference in antibiotic policies adapted in different hospitals.

Among the 40 MDR and 40 MDS *P. aeruginosa* isolates, 17 were CR (MIC between 4 and $\geq$ 16 μg/mL) and 23 were CI (MIC $\leq$2 μg/mL) strains as per the MIC detected by the agar dilution method. Earlier studies have reported that the prevalence of colistin resistance in MDR and MDS *P. aeruginosa* is 21% (16 of 75) and 12.5% (10 of 80 isolates).[6,23] Our study showed that a greater number of isolates were CI. The judicious use of antimicrobials and stringent infection regulating practices are necessary to stop the emergence of colistin resistance.

Swimming and swarming motility of *P. aeruginosa* mediated by flagella and twitching by type 4 pili are linked to virulence traits in *P. aeruginosa*. They first become sessile and adhere to surfaces to form a biofilm.[9] We found that swimming motility was higher in CI strains and swarming motility was higher in CR strains. Twitching motility was seen only in MDR strains but not in MDS strains (Table 2). Thus, a statistically significant difference was seen only in twitching motility of MDR and MDS strains. Loss of motility contributes to biofilm formation, which helps the bacteria protect itself from the effects of antibiotics and gives a survival advantage.[10] However, there was no statistically significant differences in biofilm production among the strains exhibiting and not exhibiting different types of motilities. We have not come across any published studies on the effects of different types of motilities on biofilm formation and drug resistance in *P. aeruginosa* to compare our results.

The percentages of distinct types of motilities observed in MDR and MDS *P. aeruginosa* by earlier workers were 76.5% and 88.5% for swimming, 40% and 72.1% for swarming, and 83.5% and 88.5% for twitching motilities, respectively.[18,24] However, the rate of motilities expressed by our MDR and MDS *P. aeruginosa* isolates was lower compared to earlier study results. Twelve MDR isolates from urine showed all three types of motilities. Hence, the virulence trait of these isolates needs to be explored further.

The biofilm-forming ability of *P. aeruginosa* isolates was detected by the microtiter plate method. Among the isolates from pus, 50% were moderate, 32.3% were strong, and 17.7% were weak biofilm producers. Of the urine isolates, 50% were moderate, 44.4% were strong, and 5.6% were weak biofilm producers (Table 3). A study by Abdulhaq et al.[25] reported a higher prevalence of biofilm-forming *P. aeruginosa* isolates in urine (60%) compared to pus (44.8%) isolates. Our results showed a correlation between drug resistance and strong biofilm production, which is in line with the results of Azimi et al.[16]

In *P. aeruginosa*, the *Mod A* gene plays a role in anaerobic growth, nitrate reduction, and biofilm formation.[26] The gene *PslA* has a major role in biofilm formation, which helps the bacteria tolerate antibiotics and the host immune system.[27] We found that the *Mod A* and *Psl A* genes were detected in a higher number of CI *P. aeruginosa* isolates compared to CR isolates (Table 4 and Figure 1). To the best of our knowledge, there are no published reports of *mod*A and *Psl A* gene detection in CI strains to compare our results of CI isolates of *P. aeruginosa*.

A study by Azimi et al.[16] reported the absence of the *Mod A* gene in CR strains. In our study, the *Mod A* gene was present in 25 of 34 CR isolates. Furthermore, the majority of moderate biofilm producers (exception: 2 each of MDR and MDS) and all of the strong biofilm producers irrespective of MDR or MDS had both genes. By contrast, the *Psl A* gene was absent in weak biofilm-forming CR-MDR *P. aeruginosa* isolates. Thus, we found a positive connection between the type of biofilm and the presence of *Mod A* and *Psl A* genes. Earlier studies have reported a prevalence of the *Psl A* gene ranging from 84% to 100%.[25]

Dafopoulou et al.[15] reported that CR *A. baumanii* strains show defective biofilm formation. Azimi et al.[16] substantiated the fact that colistin resistance is linked to a lower degree of biofilm formation and absence of *Mod A* gene in *P. aeruginosa* isolates. However, we found that a greater number of MDR isolates ($n = 19$) were strong biofilm producers compared to MDS isolates ($n = 9$) irrespective of whether they were CR or CI. Though all isolates formed biofilm, *Mod A* and *Psl A* genes were absent in a few of the weak and moderate biofilm-forming strains. This shows the possibility that other genes or factors may be involved in biofilm formation. A detailed investigation of these factors should be considered in future work. Because this was a laboratory-based study, we could not correlate the clinical and demographic data of the patients with the different parameters analyzed.

Conclusions

The results of this study showed that MDR isolates are strong biofilm producers compared to MDS isolates, irrespective of colistin susceptibility. Furthermore, most of the

strong biofilm-producing isolates had both *Mod A and Psl A* genes, whereas the *Psl A* gene was absent in weak biofilm forming CR MDR isolates. In conclusion, our results clearly indicate that the biofilm production of MDS isolates does not have a strong correlation with its colistin susceptibility and motivates future efforts to investigate the other genes and factors that may be involved in biofilm formation and colistin resistance in *P. aeruginosa* clinical isolates.

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Conflict of interest

The authors have no conflicts of interest to declare.

Ethical approval

This study was approved by the institutional ethics committee, Kasturba Medical College, Mangalore, Manipal Academy of Higher Education, Manipal (Reference No: IECKMCMLR-12/2020/402).

Author's contribution

BD designed the experiment; MPV performed the experiments; BD & MPV analyzed the data; MPV & BD wrote the paper and approved the final version. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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Availability of supporting data

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtumed.2022.10.012.

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