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ARTICLE

Phenotypic characteristics of environmental *Pseudomonas aeruginosa*: an *in vitro* study on epidemiological aspects

Mário Gajdács[1,*], Edit Urbán[2], Péter Pallós[1], Adrienn Márta[1], Danica Matusovits[3], Krisztina Kárpáti[4], Basem Battah[5], Helal F. Hetta[6], Ameer Khusro[7], Dani Dordevic[8], Ivan Kushkevych[9]

[1]Department of Oral Biology and Experimental Dental Research, Faculty of Dentistry, University of Szeged, Szeged, Hungary
[2]Department of Medical Microbiology and Immunology, University of Pécs Medical School, Pécs, Hungary
[3]Department of Prosthodontics, Faculty of Dentistry, University of Szeged, Szeged, Hungary
[4]Department of Orthodontics and Pediatric Dentistry, Faculty of Dentistry, University of Szeged, Szeged, Hungary
[5]Department of Biochemistry and Microbiology, Faculty of Pharmacy, Antioch Syrian Private University, Maaret Siadnaya, Syria
[6]Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt
[7]Centre for Research and Development, Department of Biotechnology, Hindustan College of Arts and Science, Padur, OMR, Chennai, India
[8]Department of Plant Origin Food Sciences, Faculty of Veterinary Hygiene and Ecology, University of Veterinary Sciences Brno, Brno, Czech Republic
[9]Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic

ABSTRACT *Pseudomonas aeruginosa* is one of the most commonly isolated species among non-fermenting Gram-negative bacteria, both from clinical samples and from environmental sources. The survival of the species in harsh conditions is ensured by the production of a protective biofilm; assessment of biofilm-forming capacity aids future pathogen eradication strategies. The aim of our present study was to assess the relationship between antibiotic resistance, biofilm-forming capacity and other phenotypic virulence factors in environmental *P. aeruginosa* isolates. One hundred and fourteen (n = 114) isolates were included in the study, which were obtained from various geographical regions and environmental origins. Antimicrobial susceptibility testing was carried out using standard protocols. Biofilm-forming capacity and pyocyanin pigment production were tested using microtiter plate-based methods. Swarming, swimming and twitching motility, and siderophore-production were assessed using agar-plate based methodologies. Resistance in environmental isolates were highest for levofloxacin/ciprofloxacin 49.12% (*n* = 56), ceftazidime 42.98% (*n* = 49) and cefepime 35.96% (*n* = 41), while lowest for colistin 0% (*n* = 0); overexpression of RND-type efflux pumps was seen in 33.33% (*n* = 33) of isolates. 21.93% (*n* = 25) met the criteria to be classified as multidrug resistant (MDR). 17.54% (*n* = 20) of isolates were weak/non-biofilm producers, while (25.45%, *n* = 29) and (57.01%, *n* = 65) were moderate and strong biofilm producers, respectively. No significant differences were noted in biofilm-formation (OD_{570} values non-MDR [mean ± SD]: 0.396 ± 0.138 vs. MDR: 0.348 ± 0.181; $p > 0.05$) or pyocyanin pigment production (OD_{686} values non-MDR: 0.403 ± 0.169 vs. MDR: 0.484 ± 0.125; $p > 0.05$) between MDR and non-MDR environmental *P. aeruginosa*. Highest motility values were observed for swarming motility, followed by swimming and twitching motility; no relevant differences ($p > 0.05$) in motility were noted in the context of MDR status or biofilm-formation in the tested isolates. *P. aeruginosa* is an opportunistic pathogen with high medical importance, being a causative agent of recalcitrant infections, which are becoming difficult to treat with the onset of MDR. Further studies are warranted to assess biofilm-forming capacity, and to provide insights into the mechanisms underlying biofilm-formation both in isolates of clinical and environmental origins.

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***Corresponding author**
E-mail: mariopharma92@gmail.com

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) – a member of the *Pseudomonadota* phylum – is one of the most commonly isolated species among non-fermenting Gram-negative bacteria, both from clinical samples and from environmental sources, which is due to the non-fastidious growth requirements and the considerable genomic plasticity characteristic for this species (Moradali et al. 2017; Nolan

et al. 2020). Members of the genus are ubiquitous, frequently isolated from aquatic environments and sediment, farming, fishing, and agricultural sites (Algammal et al. 2020; Gutiérrez-Barranquero et al. 2019). In addition, *P. aeruginosa* is an important cause of healthcare-associated infections (HAIs; such as ventilation-associated pneumonia, bacteremia, sepsis, skin and soft tissue infections, eye infections and otitis externa), which are most commonly found in severely debilitated patients, affected by innate or acquired immunosuppression, cancer, invasive surgical interventions or other chronic conditions (e.g., cystic fibrosis [CF], diabetes) (Cicek et al. 2021; Veesenmeyer et al. 2009). It has been estimated that 8-20% patients will be colonized by *P. aeruginosa* during their hospital stays (Klockgether and Tömmler 2017).

P. aeruginosa possesses a relatively large genome (~5.5–7 Mb), which allows for the possession of numerous genes encoding for virulence factors, such as a Type III secretion system, exotoxins A, S, T and U, proteases and other degrading enzymes, pigments (most notably pyocyanin, pyomelanin and pyoverdine), flagella and iron-acquisition systems (siderophores) (Bentzmann and Plésiat 2011; Pang et al. 2019). Undoubtedly, one the most relevant protective factor for pseudomonads is the production of biofilm (Ciofu et al. 2017); biofilms consist of aggregated bacterial communities (either monospecies or multispecies), embedded in a matrix of exopolysaccharides, proteins, lipids, environmental DNA (eDNA) and water, bestowing protection from harsh environmental conditions (e.g., sheer forces, drying damage), the immune system (phagocytes, immunoglobulins) and antibiotics (Azeredo et al. 2017; Lebeaux et al. 2014). Biofilm-formation contributes to the persistence of *P. aeruginosa* in strident environmental conditions and in healthcare-associated environments (e.g., water taps, respiratory tubes, dental unit water systems) (Khajezadeh et al. 2022; Maurice et al. 2018). Therefore – from the perspectives of infection prevention and control (IPC) – insights into biofilm-formation are crucial for the successful eradication of these pathogens (Spangolo et al. 2021).

Antimicrobial resistance has emerged as one of the most critical issues facing humanity in the 21st century (O'Neill 2014). *P. aeruginosa* is one of the most common multidrug-resistant (MDR) pathogens encountered in clinical practice; these infections are often difficult to treat (due to the numerous intrinsic resistance mechanisms coupled with acquired resistance genes), with limited therapeutic options for clinicians to choose from (López-Causapé et al. 2019). According to the Global Burden of Antimicrobial Resistance study, *P. aeruginosa* is among the six leading pathogens for deaths associated with MDR (Antimicrobial Resistance Collaborators 2022), while the World Health Organization (WHO) has designated carbapenem non-susceptible *P. aeruginosa* as a "Priority 1: Critical" pathogen for the development of novel antibiotics (World Health Organization 2017). With carbapenem resistance on the rise, the use of older, more toxic drugs (e.g., colistin) is often necessary, which may lead to adverse outcomes and sequelae (Jeannot et al. 2021). There has been a pronounced interest in developing alternative approaches to prevent and treat bacterial infections, including phage therapy, antimicrobial peptides, vaccine development, drug repurposing and the introduction of antimicrobial adjuvants (e.g., as anti-biofilm or anti-virulence agents) (Fayad et al. 2018; Killough et al. 2022; Lagadinou et al. 2020; Pushpakom et al. 2019; Zhang et al .2023). In the recent past, there has been an increased interest in possible association (or co-occurrence) of the MDR-phenotype and the expression of various virulence factors and/or biofilm-formation in clinically relevant Gram-positive and Gram-negative pathogens (Carcione et al. 2022; Elmouaden et al. 2019; Maione et al. 2023). As biofilms represents a tertiary form of drug resistance (by inhibiting the diffusion of antimicrobials), a possible link between the two protective mechanisms (either through genetically encoded or adaptive mechanisms of gene expression) may also have important consequences for the outcomes of infections (Aldman et al. 2023). While the number of studies in this field has increased considerably for both *P. aeruginosa* and for other biofilm-forming pathogens, the currently accumulated evidence is still controversial, as numerous publications – based on both *in vitro* and *in vivo* results – have derived markedly different conclusions (Ghasemian et al. 2023; Mirzahosseini et al. 2020; Zhao et al. 2020).

Previously, we have assessed whether the propensity to form biofilm is predicted by antimicrobial resistance in *P. aeruginosa* from both clinical (Gajdács et al. 2021) and environmental origins (Behzadi et al. 2022); our results so far have shown no significant associations between biofilm-forming capacity and the MDR-status of the isolates. Moreover, as a secondary outcome, our *in vitro* studies also highlighted that there was no association between biofilm formation, motility, or the production of various other virulence factors (Behzadi et al. 2022). To verify and complement our existing findings, the aim of our present study was to assess whether a relationship exists between antibiotic resistance, biofilm-forming capacity and other phenotypic virulence factors in a batch of environmental *P. aeruginosa* isolates. Our initial hypotheses – derived from previous findings – were the following: *(i)* environmental *P. aeruginosa* isolates are strong biofilm-producers; *(ii)* there is no significant association between the production of biofilm or pigment and MDR; *(iii)* there is no significant association between biofilm-production and the expression of other virulence factors.

Materials and methods

Sample size determination

The required sample size of environmental *P. aeruginosa* isolates was determined using the formula below (1), according to the methodology described by Thrusfield et al. (2001), where *n* was the calculated sample size, *z* was the desired confidence level (1.96), *i* was the standard sampling error (5%), while *p* was the estimated prevalence set at 5% (Odongo et al. 2020). Based on the calculation, the required sample size of *n* = 114 isolates was determined.

$$n = z^2 p (1-p)/i^2 \qquad (1)$$

Collection of isolates

A total of one hundred and fourteen (*n* = 114) isolates were included in the study, which were obtained from various geographical regions and environmental origins, i.e. from outdoor sources (e.g., agricultural sources, plants, sediments, soil and surface waters), and sources with high rates of anthropogenic presence (e.g., handles, steel and rubber surfaces). Environmental sampling procedures were carried out according to protocols previously described (Kaszab et al. 2021), between 1st of January 2020 and 1st of January 2021. As a general rule, only one *P. aeruginosa* isolate per source was included (Behzadi et al. 2022). During the experiments, *P. aeruginosa* ATCC 27853 (characterized by limited biofilm-production and MDR status), and *P. aeruginosa* PAE 170022 (characterized by strong biofilm-production and susceptibility to antibiotics) were used as control strains, which were obtained from the American Type Culture Collection (ATCC; Manassas, VI, USA) (Saeki et al. 2021). Stock cultures were stored at - 80 °C in a cryopreservation medium (700 µL tryptic case soy broth + 300 µL 50% glycerol) until further use.

Microbial identification procedures

P. aeruginosa isolates were identified to the species level before inclusion in further experiments; identification was carried out by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF MS). The instrument used was the MicroFlex MALDI Biotyper (Bruker Daltonics, Bremen, Germany), while spectra analysis was performed with the MALDI Biotyper RTC 3.1 software and the MALDI Biotyper Library 3.1 (Bruker Daltonics, Bremen, Germany). Detailed technical characteristics of the mass spectrometry measurements were described elsewhere (Schubert and Kostrzewa 2017). Reliable species-level identification was accepted in the case of a log(score) value ≥2.30.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed using the standard disk diffusion method (Oxoid, Basingstoke, UK) on Mueller-Hinton agar plates (bioMérieux, Marcy-l'Étoile, France) including anti-pseudomonal cephalosporins (ceftazidime, cefepime), anti-pseudomonal carbapenems (imipenem, meropenem), fluoroquinolones (ciprofloxacin, levofloxacin), aminoglycosides (amikacin, gentamicin) and colistin. With the exception of colistin, interpretation of the AST results was based on the standards and breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) v. 11.0 (European Committee on Antimicrobial Susceptibility Testing 2022). Results indicating "susceptible, increased exposure (I)" were grouped with and reported as susceptible (S) (European Committee on Antimicrobial Susceptibility Testing 2019). Susceptibility to colistin was assessed according to the provisional breakpoints as advised by Galani et al. (Galani et al. 2008), using 10 µg colistin-containing disks (i.e. inhibition zones ≥ 14 mm were considered susceptible). Classification of the isolates as MDR (resistance to at least one agent in ≥ 3 antibiotic groups) was based on the recommendations of Magiorakos et al. (2012).

Detection of efflux pump overexpression using phenotypic methods

The overexpression of resistance-nodulation-division-type (RND) efflux pumps was tested if ciprofloxacin-resistance was noted based on the disk diffusion test. The assay was carried out using a phenylalanine-arginine β-naphthylamide (PAβN)-based agar dilution method, described previously (Khalili et al. 2019). A two-fold decrease in ciprofloxacin MICs (measured by E-tests; Liofilchem, Roseto degli Abruzzi, Italy) in the presence of PAβN, compared to the MIC values without the inhibitor, was considered positive for efflux pump overexpression (Gajdács 2020; Khalili et al. 2019). *P. aeruginosa* ATCC 27853 was used as a control strain.

Assessment of biofilm-forming capacity

Biofilm-formation in environmental *P. aeruginosa* was assessed using a microtiter-plate based method, as previously described (Ramos-Vivas et al 2019). Briefly, overnight *P. aeruginosa* cultures, grown on Luria–Bertani [LB] agar, were inoculated into 5 mL of LB-broth and incubated overnight at 37 °C. The next day, 20 µL of bacterial suspension (set at 10^6 CFU/mL) and 180 µL of LB-broth were transferred onto 96-well flat-bottomed microtiter plates to a final volume of 200 µL. Following a 24 h incubation period at 37 °C, supernatants were discarded, and the wells were washed three times using 200 µL of phosphate-buffered saline (pH set at 7.2). Following this

washing step, the wells were fixed with 250 μL of methanol (Sigma-Aldrich, St. Louis, MO, USA) for 10 min, and stained with a 1.0% crystal violet (CV; Sigma-Aldrich, St. Louis, MO, USA) solution for 15 min. The CV dye was then discarded, and the wells were washed three times with purified water to remove excess stain. The wells' contents were solubilized in 250 μL of 33% v/v% glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA), and a microtiter plate reader was used to measure absorbance at 570 nm (OD_{570}). Results of the experiments originate from three independent measurements. Interpretation of biofilm-forming capacity was based on the guidelines set by Ansari et al. (2014): isolates with OD_{570} values < 0.12 were classified as weak/non-biofilm producers, OD_{570} values between 0.12–0.24 were classified as moderate biofilm producers, while OD_{570} values > 0.24 were classified as strong biofilm producers, respectively.

Assessment of bacterial motility

Motility assays on environmental *P. aeruginosa* were carried out in an agar-based assay: Petri dishes containing Tryptic Soy Agar were inoculated with the tested isolates, where media contained different agar concentrations (0.3% for swimming motility, 0.8% for swarming motility, and 2.0% for twitching motility, respectively) for different assays (Ha et al. 2014). Overnight bacterial cultures (set at a density of 10^5 CFU/mL) were transferred into the agar medium by puncture using a pipette tip (at 1/2 depth for swimming and swarming motility and at full depth for twitching motility) (Ha et al. 2014). Following inoculation at 37 °C for 24 h (swimming and swarming motility) or 48 h (twitching motility), diameters of bacterial growth (in mm) were recorded; in the case of swimming and swarming motility, the measurements were made directly, while in case of twitching motility, the agar layer was removed and the bottom of the plates was stained directly with 0.01% CV solution (Ha et al. 2014; Markwitz et al. 2021; Turnbull and Whitchurch 2014). Results of the experiments originate from three independent measurements.

Assessment of pyocyanin pigment production

Pyocyanin pigment production was assessed in 24-well tissue culture plates (Sarstedt, Nümbrecht, Germany) containing an overnight bacterial culture of *P. aeruginosa*, incubated at 37 °C for 48 h. Following the incubation period, each bacterial suspension was collected in an Eppendorf tube and centrifuged at 10 000 rpm (Das and Manefield 2012; Markwitz et al. 2021). The supernatants were transferred to 96-well microtiter plates, after which, the absorbance for pyocyanin (OD_{686}) was measured using a microtiter plate reader. Results of the experiments originate from three independent measurements.

Statistical analysis

Descriptive statistical analysis (means with ranges and percentages) was carried out using Microsoft Excel 2013 (Microsoft, Redmond, WA, USA). Normality testing was performed by the graphical method and the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) with Tukey's post hoc test was used to compare growth zones (for swimming, swarming and twitching motility) between different biofilm-producing *P. aeruginosa* populations. An independent sample t-test was used to compare biofilm-forming capacity and pyocyanin-production between MDR and non-MDR *P. aeruginosa*. Inferential statistical analyses were performed using SPSS software version 22.0 (IBM, Armonk, NY, USA). $p < 0.05$ was considered statistically significant.

Results

Antimicrobial resistance and efflux pump overexpression in environmental P. aeruginosa

Rates of non-susceptibility in environmental *P. aeruginosa* were as follows (in increasing order): colistin 0% ($n = 0$), meropenem 14.04% ($n = 16$), imipenem 14.91% ($n = 17$), amikacin 16.67% ($n = 19$), gentamicin 17.54% ($n = 20$), cefepime 35.96% ($n = 41$), ceftazidime 42.98% ($n = 49$), levofloxacin 49.12% ($n = 56$) and ciprofloxacin ($n = 56$). Out of these isolates 21.93% ($n = 25$) met the criteria to be classified as MDR. When phenotypic ciprofloxacin resistance was noted, the overexpression of RND-type efflux pumps was also tested; based on the plate-based assay, 67.86% ($n = 38$ out of 56 isolates; 33.33% overall) of isolates were positive.

Biofilm-forming capacity, and phenotypic virulence factors in environmental P. aeruginosa

Based on the results of the CV-based quantitative biofilm-formation assay (where OD_{570} values were measured), isolates were classified as follows: non-producing/weak biofilm-producers (17.54%, $n = 20$; $OD_{570} < 0.12$), moderate biofilm-producers (25.45%, $n = 29$; OD_{570}: 0.12–0.24) and strong biofilm-producers (57.01%, $n = 65$; $OD_{570} > 0.24$), respectively. The positive control isolate PAE 170022 showed OD_{570} values [mean ± SD] of 0.462 ± 0.074, while the negative control isolate ATCC 27853 showed OD_{570} values of 0.096 ± 0.011, respectively. During the comparison of biofilm-formation between MDR and non-MDR environmental *P. aeruginosa*, no significant differences were noted, based on the measured OD_{570} values (OD_{570} values non-MDR [mean ± SD]: 0.396 ± 0.138 vs. MDR: 0.348 ± 0.181; $p > 0.05$).

The results of the motility assays (expressed in mm) – with regards to the groups based on biofilm-forming

capacity – are summarized in Table 1.; highest motility values were observed for swarming motility, followed by swimming and twitching motility. Significant differences were not found in motility between the isolates of different biofilm-forming capacities (Table 1.). Similarly, when comparing motility in the context of the MDR status of the isolates, no significant differences were found (swarming: non-MDR: 30.31 ± 11.99 vs. MDR: 32.17 ± 12.23; $p > 0.05$; swimming: non-MDR: 26.92 ± 9.12 vs. MDR: 25.26 ± 9.21; $p > 0.05$; twitching: non-MDR: 12.03 ± 4.58 vs. MDR: 11.25 ± 5.03; $p > 0.05$). Pyocyanin production (measured as OD_{686}) was also assessed between MDR and non-MDR *P. aeruginosa* isolates, where no statistical association was shown (MDR: 0.484 ± 0.125 vs. non-MDR: 0.403 ± 0.169; $p > 0.05$).

Discussion

In the present study, the biofilm-forming capacity and pigment-production of environmental *P. aeruginosa* were assessed, with special regard to the possible relationship of the MDR-phenotype and virulence-factor expression. The importance of studies on both clinical and environmental *Pseudomonas* spp. have been highlighted with the introduction of the "One Health" concept, as environmental pseudomonads were suggested as viable reservoirs of antibiotic resistance genes, and their role in zoonotic infections (e.g., in birds) has also been described (Algammal et al. 2022; Balcázar et al. 2015; El-Ghany 2021). In addition, the prevalence of *P. aeruginosa* infections is on the rise, together with the number of immunocompromised individuals, and patients undergoing invasive medical interventions (Sanya et al. 2023). Most of the isolates (82.46%) were biofilm-producers, with almost 60% being strong biofilm-producers; the present batch of isolates had the highest rate of biofilm-producers, when compared to our previous studies on environmental (Behzadi et al. 2022) or clinical isolates (Gajdács et al. 2021). On the other hand, antimicrobial resistance rates and percentage of MDR isolates was lowest in our current study (with highest resistance rates found in clinical isolates for most anti-pseudomonal antibiotics (Gajdács et al. 2021)), although the common tendencies (highest resistance for fluoroquinolones, followed by cephalosporins, aminoglycosides and carbapenems, while most isolates were susceptible to colistin) were similar in all reports (Behzadi et al. 2022; Gajdács et al. 2021). In line with our previous experimental results for *P. aeruginosa*, we could not detect significant associations between the MDR-phenotype and biofilm-forming capacity in the tested isolates. Furthermore, no differences in motility (with swarming motility being the most pronounced, followed by swimming and twitching motility, as seen previously (Behzadi et al. 2022; Gajdács et al. 2021)) and pigment production were shown either. Interestingly, in our study with clinical *P. aeruginosa* isolates, pyocyanin production was higher among MDR *P. aeruginosa*.

Microbial biofilm-production is one of the most effective protective mechanisms against harsh environmental conditions and the immune system of the host (Sharma et al. 2023); additionally, due to the heterogeneous composition of these biofilms, the complex interplay of multiple drug resistance mechanisms (i.e. "biofilm resistance", which is mediated by the expression of various genes in the cells) may result in minimum inhibitory concentrations (MICs) 100-10000-times higher, compared to those against planktonic bacteria (Donadu et al. 2018). The results of Arslan and co-workers have shown that sub-MIC treatment of *P. aeruginosa* with various antibiotics (ciprofloxacin, fosfomycin, piperacillin/tazobactam and tobramycin) resulted in increased expression of biofilm-specific resistance genes and motility (Arslan et al. 2023). Hindering adherence to surfaces (a critical initial step in biofilm-formation) and/or biofilm-maturation by various molecules (either synthetic or from natural sources) is considered a viable strategy for future drug development; these compounds may act through a variety of mechanisms, such as leading to changes in the bacterial metabolism, inhibition of quorum sensing (QS) and inhibition of efflux pumps, among others (Talebi-Taher et al. 2016). For example, Peppoloni et al. (2023) showed that the phenolic compounds found in pomegranate peel extract have anti-biofilm properties, and they also hinder autoinducer (AI) signaling in *P. aeruginosa*, thereby leading to attenuated

Table 1. Relationship between biofilm-production and motility in environmental *P. aeruginosa*.

Virulence factor	Weak/non-biofilm producers 17.54% (*n* = 20)	Moderate biofilm producers 25.45% (*n* = 29)	Strong biofilm producers 57.01% (*n* = 65)	Significance
Swimming motility (mm) (mean ± SD)	24.92 ± 10.11	25.78 ± 9.34	27.16 ± 11.93	$p > 0.05$
Swarming motility (mm) (mean ± SD)	33.01 ± 12.30	31.82 ± 10.78	31.11 ± 9.89	$p > 0.05$
Twitching motility (mm) (mean ± SD)	11.76 ± 4.92	11.34 ± 5.14	12.76 ± 6.31	$p > 0.05$

virulence. Likewise, the experiments of Tsavea et al. (2023) highlighted the potential of honeys from Mt. Olympus as anti-virulence compounds, inhibiting pigment production, motility, and biofilm-formation in the tested isolates. Di Bonaventura and co-workers performed the screening of >3,000 FDA-approved drug molecules to identify novel therapeutic leads with antimicrobial and anti-biofilm activity against *P. aeruginosa*, in conditions relevant to CF-infected lungs (Di Bonaventura et al. 2023); in their study, Ebselen (an anti-inflammatory and antioxidant compound), tirapazamine, carmofur, 5-fluorouracil (antitumor agents) and tavaborole (antifungal) showed relevant antimicrobial properties in time-kill assays, on the other hand, only tirapazamine and tavaborole were effective in dispersing preformed biofilms. The importance of the *de novo* pyrimidine synthesis pathway (which is inhibited by numerous anticancer medication) in intact bacterial virulence for *P. aeruginosa* has also been highlighted by Niazy et al. (2022). On the other hand, Valentin and co-workers proposed a different approach, where a public *P. aeruginosa* transposon insertion library was utilized, to screen for biofilm-relevant genes: with this approach, potential future molecular targets could be identified to aid advances in drug development (Valentin et al. 2023).

The meta-analysis of Mirzahosseini et al. (2020) aimed to summarize the available evidence on the association between biofilm-formation and MDR in clinical *P. aeruginosa* published between 2000 and 2019: based on the pooled data, 86.5% (95% CI: 79.0-91.6%) of isolates produced biofilm, out of which, 51.0% were strong biofilm-producers; highest rates of resistance were recorded against anti-pseudomonal β-lactams (namely piperacillin-tazobactam, 90%), and the prevalence of virulence genes were >90% in most studies. Overall, the meta-analysis has shown that the rate of biofilm-production was higher in MDR isolates, with a significant correlation identified in >50% of included studies (Mirzahosseini et al. 2020). This positive association was further underlined in the recent publication of Baskan et al. (2023), the report of Zahedani et al. (2021) (where an association was found between efflux pump overexpression and biofilm-formation), and the experiments of Karami et al. (2018) (where the MDR status was significantly more common in strong biofilm-producers in case of both clinical and environmental *P. aeruginosa*). In line with our current findings, the results of Choy et al. (2008) (involving isolates from keratitis), Bahador et al. (2019) (involving isolates from keratitis), and Radó et al. (2017) (involving environmental isolates) did not show any relevant correlation between drug resistance, biofilm-formation, and the detection of specific virulence genes. In contrast, several studies have described as inverse relationship between the presence of resistance determinants and biofilm-formation: for example, Edward and co-workers showed that in clinical *P. aeruginosa*, presence and expression of specific virulence genes was associated with susceptibility to various antimicrobials (e.g., ceftazidime and aztreonam susceptibility) (Edward et al. 2023). Gallant et al. (2005) found that isolates carrying the β-lactamase TEM-1 β-lactamase have impaired adhesive capacity, which is crucial in the initial stages of biofilm-formation. While in the study of Yamani et al. (2021), upregulation of biofilm and virulence-associated genes was seen in susceptible isolates, compared to their MDR counterparts.

Conclusions

P. aeruginosa is an opportunistic pathogen with high medical importance, being a causative agent of recalcitrant infections, often affecting the most debilitated patients. *P. aeruginosa* infections are becoming difficult to treat with the onset of MDR, with isolates susceptible only to last-resort drugs with severe adverse effects (e.g., colistin) or to novel antibiotics, which are often not available in developing countries. Based on our present findings – which were concurrent with our previous results – no relationship was found between biofilm-forming capacity, pigment production, motility, and drug resistance in the tested isolates; however, no sweeping conclusions can be made based on the current evidence. Further studies are warranted to assess biofilm-forming capacity, and to provide insights into the mechanisms underlying biofilm-formation in *P. aeruginosa*, both in isolates of clinical and environmental origins.

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