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Virulence Factors, Drug Resistance and Biofilm Formation in *Pseudomonas* Species Isolated from Healthcare Water Systems

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Abstract

Pseudomonas aeruginosa is a frequent causative agent of healthcare-associated diseases, but recently, other members of the *Pseudomonas* genus have been recognized to cause human colonization and infection. Since the aquatic environment could be an important source of contamination, we studied the drug resistance and virulence profiles in *Pseudomonas* species isolated from healthcare water systems. 17 *Pseudomonas* spp. out of 57 were randomly selected and their drug resistance and virulence profiles were later evaluated. Based on the positivity to the tests, the adhesion capability and biofilm formation on polystyrene and glass surfaces were studied in 6 strains, each belonging to different species. Six *Pseudomonas* strains (35%) were α-hemolytic, nine (53%) showed a positivity to the gelatinase test, and *P. acidovorans* 2R only was capable to degrade DNA. All *Pseudomonas* strains presented urease activity and the production of siderophores was widely observed (64,7%). Most of the strains showed one of the three types of motilities, 15 *Pseudomonas* (88.23%) resulted bacteriocin producers and all strains were resistant to one or more antibiotics. Lastly, among the six selected strains, *P. aeruginosa* 98.5 and *P. fluorescens* 97.4 were the best biofilm producers. Our study has highlighted how the majority of isolates shows biological characteristics that contribute to the pathogenicity of *Pseudomonas*. These features emphasize the virulence potentiality of other members of the *Pseudomonas* genus besides *Pseudomonas aeruginosa*, making them potentially pathogenic, especially against immunocompromised individuals.

Introduction

In many healthcare facilities, the water distribution system results colonized by a variety of microorganisms, most of which are of the *Pseudomonas* species, opportunistic pathogens able to infect immunocompromised individuals. *Pseudomonas aeruginosa* is the major specie responsible for healthcare-associated diseases, such as urinary tract infections (UTI), pneumonia, and septicemia, but other members of the *Pseudomonas* genus, usually considered saprophytic species, can cause human colonization or infection [1–3]. *P. fluorescens* has been reported to be responsible for bacteremia in humans and has also been isolated from clinical samples of mouth, stomach, and lungs [4]. *P. putida* has been documented as the causative agent in several nosocomial infections, mostly in immunocompromised patients [5–8]. Non-*Ps. aeruginosa Pseudomonas* (NPAP) strains resulted implicated in adult bacterial meningitis in a retrospective study [9].

Since both *Pseudomonas aeruginosa* and other *Pseudomonas* strains may be present in humans as a mere colonizer, and the isolation of these microorganisms from clinical samples does not necessarily implicate an infection, it is necessary to aquire more exhaustive information on the presence of virulence factors in all members of the *Pseudomonas* genus, in order to determine the extent and severity of the disease they may cause. *Pseudomonas'* ability to produce hemolysin, gelatinase, urease, and bacteriocins must be considered an important asset to their pathogenicity. Furthermore, the ability to form biofilm, a microbial structure that protects bacteria from unfavorable environmental conditions [10–15], is a critical feature already reported in a lot of studies carried out on *Pseudomonas* isolated from environmental, food, and clinical samples. The microorganisms of the genus *Pseudomonas* are known for their remarkable ability to adhere and form biofilms thanks to surface structures such as the polar flagella, responsible for the swimming and twitching movements. Mobility is a characteristic that allows significant advantages, such as greater efficiency in nutrient

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acquisition and surface colonization, the latter also due to an increased cell adhesion during the initial phase of biofilm development and a subsequent persistence of the same.

Lastly, even though most of the other NPAP are usually considered to possess a lower degree of pathogenicity, if compared to *Pseudomonas aeruginosa*, when they invade the human organism their antibiotic susceptibility results reduced, and they often exhibit multiple resistance to antibiotics. The presence of plasmids harboring the genes that encode antibiotic resistance factors in most NPAP species, as well as their capability to transfer by conjugation to other microorganisms in hospital environments, has been reported [16–18]. Given that all *Pseudomonas* species are widely found in aquatic environments, the spread of multidrug-resistant bacteria through hospital water is a cause of concern. Water systems have been reported to contribute to *P.* aeruginosa transmission in healthcare settings [19], and recent studies demonstrated that other members of the genus *Pseudomonas* living in drinking waters can be important reservoirs of antibiotic resistance [20].

Since the aquatic environment could be an important source of contamination, we investigated the presence of *Pseudomonas* species in healthcare water systems. Therefore, given that antibiotic resistance and other expression of virulence traits could justify the increased pathogenicity of *Pseudomonas* spp other than *P. aeruginosa*, we studied the drug resistance and virulence profiles of 17 randomly selected out of 57 *Pseudomonas* spp. isolates. Based on the positivity to the tests, six strains, each belonging to different species, were selected to evaluate the adhesion capability and biofilm formation on polystyrene and glass surfaces.

Methods

Culture Conditions and Bacteria Identification Methods

Pseudomonas strains, coming from different wards of Modena S. Agostino-Estense hospital (Modena, Italy), were isolated and identified from different healthcare water systems (Modena, Italy), according to the standardized procedure UNI EN ISO 16266 [21]. The strains were grown in Tryptic Soy Broth (TSB, bioMérieux, Florence, Italy) supplemented with 0.6% yeast extract (TSB-YE) (bioMérieux, Florence, Italy) and kept at 30 °C for 24 h. The isolates were screened by colony morphology, diffusible pigment production, Gram stain, oxidation/fermentation of glucose, motility, cytochrome oxidase (N,N-dimethyl-p-phenylenediamine dihydrochloride, Sigma Chemical, St. Louis, MO, USA), and biochemically identified by Vitek 2 system (bioMèrieux, Florence, Italy). The bacterial identification was definitively confirmed by using 16S rRNA sequence. Genomic DNA from each strain was first extracted with the Genomic DNA DNeasy Blood & Tissue Kits (Qiagen, Milan, Italy), and amplification of the 16S rRNA gene was carried out using universal primers: (16SF 5′-AGAGTTTGATCCTGGCTCAG-3′; 16SR 5′-CTACGGCTACCTTGTTACGA-3′) [22] and sequenced. All sequences were then compared to those in the GenBank database using the BLAST program [23] to confirm the taxonomic identification at species level.

All the strains were stored in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KH_2PO_4 with 1L of distilled water) supplemented with 30% (vol/vol) glycerine at − 80 °C.

Virulence Tests

With the purpose of evaluating as many biological characteristics as possible, we have chosen to conduct a preliminary evaluation of virulence factors in a representative sample of 17 randomly selected *Pseudomonas*. Hemolysin, gelatinase, DNase, urease, and siderophore production were evaluated by spotting the plates added with the specific media with 10 µL of *Pseudomonas* spp. suspension, cultured in Tryptic Soy broth (TSB, bioMérieux, Florence, Italy) at 30 °C for 48 h. For the hemolysin production, Blood Agar Base (bioMérieux, Florence, Italy) containing 5% of defibrinated horse blood (Oxoid, SpA, Milan, Italy) was used. After incubation, the hemolytic activity was determined by the appearance of a clear zone of hemolysis (β-hemolysis), a partial and greening hemolysis zone (α-hemolysis), or no activity (γ-hemolysis) around the spots. Gelatinase production was assessed by inoculation of the strains in a Nutrient broth containing 10% gelatin. Positive gelatinase was recorded as degradation of the gelatin to liquid. For detection of DNase activity, the isolates were cultured on DNase agar (bioMérieux, Florence, Italy) supplemented with 0.5% yeast extract (bioMérieux, Florence, Italy), and a clearing zone around the inoculum was registered as a positive reaction. For the urease production, a Urea Agar slope (bioMérieux, Florence, Italy) was inoculated with *Pseudomonas* spp. suspension and a positive result was recorded when the color changed from yellow to pink. Siderophore production was screened as described by Schwyn and Neilands [24] on chrome azurol S (CAS) agar plate. The development of yellow or orange halo zone around the spot due to the removal of iron from the dye complex by siderophore was considered as positive for siderophore production. Lastly, the swimming, swarming, and twitching motilities of the 17 selected *Pseudomonas* isolates were investigated. All strains were grown overnight and inoculated onto plates added with the following media (all from Thermo Fisher Diagnostics, Milan, Italy): (i) swim plate (triptone 1%, NaCl 0.5%, agar 0.3%), (ii) swarm plate (nutrient broth 8 g/L, glucosio 5 g/L, agar 0.5%), and (iii) twitch plate (LB broth solidified with 1% wt/vol agar). For

the swimming and swarming assays, the agar media were inoculated with a sterile toothpick, whereas for the twitching assay, strains were stabbed through a 3 mm layer to the bottom of the Petri dish [25–27]. All cultures were incubated at 30 °C for 72 h. For the swimming and swarming assays, the mobility was determined by measuring the diameter of the circular turbid zone formed by the cells migrated from the injection site. The widespread of the hazy zone of growth at the interface between the agar and the polystyrene surface was a measure of twitching motility. The strains were classified as follows: non-motile (diameter of growth less than 0.5 cm), motile (+ diameter 1–2 cm), and svery motile (++ diameter up to 2 cm).

Detection of Bacteriocinogenic Activity

All the 17 isolates were screened for bacteriocin production using a modified deferred antagonism method [28], using two Gram-positive (*Staphylococcus aureus* ATCC 6538 *and Enterococcus faecalis* ATCC 29212*)* and two Gram-negative (*Escherichia coli* ATCC 25922 and *Salmonella enteritidis* ATCC 6619) pathogens as indicators. A non-bacteriocinogenic *P. aeruginosa* ATCC 9027 strain was used as negative control. The indicators were cultured in Tryptic Soy Broth (TSB, Biomerieux, Italy) and incubated at 37 °C for 24 h. To eliminate inhibition due to hydrogen peroxide production, a first incubation was performed anaerobically. The bacteriocin production was revealed by a clear zone of inhibition in the indicator lawn around the spot of the producer and it was quantified by measuring the size of the haloes:— no zone of inhibition; +, 5 mm < zone < 10 mm; ++, 10 mm < zone < 15 mm.

Antibiotic Resistance Determination

The antibiotic resistance was assessed by the broth microdilution method, following the Clinical Laboratory Standards Institute guidelines (CLSI) [29], to determine the minimum inhibitory concentrations (MICs). The following 11 antimicrobials (all from Sigma Chemical Co.) were tested: piperacillin/tazobactam, piperacillin, ceftazidime, cefepime, meropenem, imipenem, colistin, aztreonam, ciprofloxacin, amikacin, and gentamicin (all from Sigma Chemical, St. Louis, MO, USA). The European Committee on Antimicrobial Susceptibility Testing (EUCAST 2018) guidelines [30] were used for the susceptibility categorization.

Plasmid DNA Analysis

For plasmid analysis, plasmid DNA was obtained using the QIAGEN plasmid Midi kit (Qiagen, Milan, Italy), following the manufacturer's instructions. DNA plasmid was analyzed in 0.7% agarose gel electrophoresis at 3.5 V/cm for 8 h in a Tris–acetate buffer. Purified plasmids of *Escherichia coli* V517 [31] were used as size reference plasmids for molecular weight determinations.

Biofilm Formation Assay

Biofilms produced by the species *P. aeruginosa* 98.5*, P. fluorescens* 97.4*, P. acidovorans* 103.2*, S. maltophilia* 102.1*, P. putida* 96.3*, and P. putrefaciens* 98.6 were grown on 12-well polystyrene microtiter plates and glass coupons, the latter being previously sterilized and placed in the microtiter plates wells. Three milliliters of purified bacterial suspensions in synthetic tap water (STW) was added to each well, to yield a final count of about 1×10^6 cfu/ml, and incubated for 9 days at 30° C. After 3, 6 and 9 day, wells and coupons were rinsed three times with 1 ml of sterile STW to eliminate non-adhered cells, and biofilm removed by scraping the whole surface of each well bottom. The biofilm suspensions were transferred to sterile tubes containing 10 mL of STW and vortexed for 30 s. Tenfold dilutions of the biofilm suspensions were plated (10 µl) on Tryptic Soy Agar (TSA, bioMérieux, Florence, Italy) supplemented with 0.6% yeast extract (TSB-YE) (bioMérieux) and kept at 30 °C for 18 h. The experiments were performed in triplicate (three wells for each sample) and the results were expressed as colony-forming units (CFU) per cm^2. The arithmetic means, expressed as log bacterial count, were plotted against incubation time and the standard deviation was reported as error bars.

GenBank Accession Numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences strains are as follows: KC713609.1 *for P. aeruginosa*, MH547417.1 for *P. fluorescences*, KC292489.1 for *P. acidovorans*, EF427732.1 for *S. maltophilia,* and HQ007351.1 for.P. *putrefaciens.*

Results

Identification of Isolated Pseudomonas

A total of 57 *Pseudomonas* spp were isolated from different water systems in healthcare setting and identified by biochemical properties and 16S rRNA gene sequences as follows: *P. aeruginosa* 32 (55,93%), *P. acidovorans* 14 (25,42%), *S. maltophilia* 5 (8,47%), *P. putida* 3 (5,08%), *P. putrefaciens* 2 (3,39%), and *P. fluorescens* 1 (1,7%).

Virulence Factors in Representative Pseudomonas Species

As shown in Table 1, only six *Pseudomonas* strains (35%) were found α-hemolytic. Nine strains (53%) showed a positivity to the gelatinase test, as demonstrated by the complete liquefaction of the media after 48 h incubation. All *Pseudomonas* strains presented urease activity and, among all the bacteria studied, only *P. acidovorans* 21.2 showed, around the growth spot, a transparent halo due to the degradation of DNA, thus showing a positive result for this test. The production of siderophores was present in most of the *Pseudomonas* isolates: 11 strains (64.7%) have demonstrated the capability to synthesize and secrete siderophores due to the impossibility of using iron from the environment. Lastly, all the *P. acidovorans* species were negative to the motility tests. Similar results emerged for *P. putida* 11.2, *P. putida* 96.3, *P. malthophila* 102.1, and *P. aeruginosa* 96.2, even if these three last species showed only swimming, swarming, and twitching activities, respectively. Apart from *P. aeruginosa* 104.1, the remaining strains resulted endowed with almost all the motility properties.

Production of Bacteriocins Among Representative Pseudomonas Species

Out of 17 *Pseudomonas,* 15 (88.23%) resulted bacteriocin producers (Fig. 1 and Table 2). The antibacterial activity was expressed against Gram-positive pathogens only, whereas no activity was observed against Gram-negative bacteria.

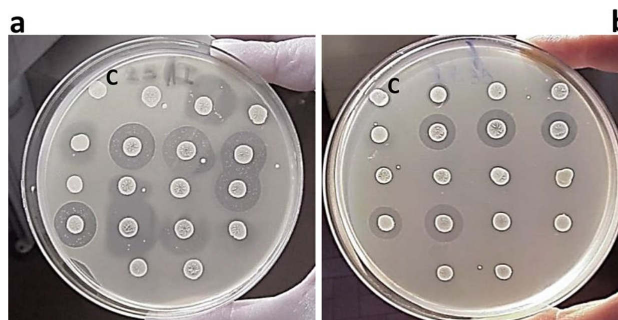

Fig. 1 Antibacterial activity of the 17 selected strains against the two Gram-positive strains *Staphylococcus aureus ATCC 6538* (**a**) and *Enterococcus faecalis* ATCC 29212 (**b**). No antibacterial activity was found against the two Gram-negative strains used in the study. C: non-bacteriocinogenic strain *P. aeruginosa* ATCC 9027 used as control. The bacteriocin production was quantified by measuring the size of the haloes: − no zone of inhibition; +, 5 mm < zone < 10 mm; ++, 10 mm < zone < 15 mm

Table 1 Virulence factors found in *Pseudomonas* species isolated from healthcare water systems

	Urease	Hemolysin	DNase	Gelatinase	Siderophore	Swimming 72 h	Swarming 72 h	Twitching 72 h
*P. acidovorans*103.2	+	−	−	−	−	−	−	−
P. acidovorans 5.3	+	−	−	−	+	−	−	−
P. acidovorans 21.2	+	+ (α)	+	−	+	−	−	−
P. putrefaciens 10.1	+	+ (α)	−	−	−	−	−	−
*P. aeruginosa*106.4	+	−	−	+	+	+	++	−
P. aeruginosa 105.3	+	−	−	+	+	++	+	+
P. aeruginosa 104.2	+	−	−	+	+	++	+	+
P. aeruginosa 104.1	+	+ (α)	−	+	+	+	−	−
P. aeruginosa 98.5	+	+ (α)	−	+	−	++	++	++
P. aeruginosa 95.1	+	−	−	+	+	++	++	−
P. aeruginosa 95.1	+	+ (α)	−	+	+	++	++	++
P. aeruginosa 84.3	+	−	−	−	−	−	−	+
P. aeruginosa 96.2	+	−	−	−	−	−	+	++
P. aeruginosa 98.6	+	−	−	+	+	++	++	++
P. fluorescences 97.4	+	+ (α)	−	+	+	+	−	−
S. maltophilia 102.1	+	−	−	−	+	−	+	−
P. putida 96.3	+	−	−	−	−	−	−	−

Urease, hemolysin, DNase, gelatinase, and siderophore production (+)/absence (−). *α* = alpha hemolysis

Motility assay. The strains were classified as: non-motile (− diameter of growth less than 0.5 cm), motile (+ diameter 1–2 cm), and very motile (++ diameter up to 2 cm)

Table 2 Antibacterial activity detected in *Pseudomonas* strains using a modified deferred antagonism method against two Gram-positive and two Gram-negative pathogens as indicators

	Escherichia coli ATCC 25922	*Salmonella enteritidis* ATCC 6619	*Staphylococcus aureus* ATCC 6538	*Enterococcus faecalis* ATCC 29212
P. acidovorans 103.2	−	−	−	+
P. acidovorans 5.3	−	−	−	+
P. acidovorans 21.2	−	−	+	−
P. putrefaciens 10.1	−	−	−	−
P. aeruginosa 106.4	−	−	++	−
P. aeruginosa 105.3	−	−	++	−
P. aeruginosa 104.2	−	−	+	−
P. aeruginosa 104.1	−	−	++	+
P. aeruginosa 98.5	−	−	++	+
P. aeruginosa 95.1	−	−	++	+
P. aeruginosa 84.3	−	−	++	−
P. aeruginosa 96.2	−	−	−	−
P. aeruginosa 98.6	−	−	−	+
P. fluorescens 97.4	−	−	++	−
S. maltophilia 102.1	−	−	+	−
P. putida 96.3	−	−	++	−
P. putida 11.2	−	−	+	−

− No zone of inhibition; +, 5 mm < zone < 10 mm; ++, 10 mm < zone < 15 mm

Antibiotic Resistance of Representative Pseudomonas Species

Table 3 shows the MIC of the isolates. All strains were resistant to gentamicin (76.5%) and amikacin (35.3%). Few isolates were resistant to meropenem (5.89%) and ceftazidime (17.64%) and no strains were resistant to high levels of ciprofloxacin, piperacillin, piperacollin/tazobactam, aztreonam, cepefime, and colistin.

Table 3 Antimicrobial activity of antibiotics against different *Pseudomonas* species

	MIC values (μg/mL)										
	PI/TZP	PIP	CAZ	FEP	MEM	IMP	CS	ATM	CIP	AMK	GE
P. acidovorans 103.2	> 1	< 1	< 1	> 1	< 1	4	< 1	4	< 1	32	64
P. acidovorans 5.3	> 1	2	< 1	> 1	< 1	1	1	2	< 1	> 128	> 128
P. acidovorans 21.2	> 1	< 1	< 1	> 1	< 1	1	> 1	2	< 1	8	16
P. putrefaciens 10.1	> 1	< 1	< 1	1	< 1	2	1	4	< 1	8	2
P. aeruginosa 106.4	> 1	8	4	2	< 1	1	> 1	4	4	8	16
P. aeruginosa 105.3	2	8	2	> 1	4	1	> 1	1	2	8	> 128
P. aeruginosa 104.2	2	2	2	> 1	0.5	1	> 1	2	< 1	16	16
P. aeruginosa 104.1	8	< 1	> 1	> 1	1	1	1	4	< 1	8	16
P. aeruginosa 98.5	> 4	4	4	> 1	4	1	> 1	4	4	16	16
P. aeruginosa 95.1	4	2	2	> 1	< 1	> 1	> 1	8	< 1	16	> 128
P. aeruginosa 84.3	8	2	4	1	1	1	2	8	< 1	8	16
P. aeruginosa 96.2	8	2	< 1	1	8	2	1	4	< 1	8	2
P. aeruginosa 98.6	8	2	< 1	2	2	2	1	4	< 1	8	8γ
P. fluorescens 97.4	4	2	< 1	2	1	1	1	4	< 1	84	16
S. maltophilia 102.1	8	4	1	1	8	2	1	4	4	> 128	32
P. putida 96.3	8	8	8	2	2	2	1	2	< 1	> 128	> 128
P. putida 11.2	4	< 1	< 1	2	< 1	1	2	4	< 1	4	1

PIP/TZP piperacillin/tazobactam, *PIP* piperacillin, *CAZ* ceftazidime, *FEP* cefepime, *MEM* meropenem, *IPM* imipenem, *CS* colistin, *ATM* aztreonam, *CIP* ciprofloxacin, *AMK* amikacin, *GEN* gentamicin

Plasmid DNA Found in Representative Pseudomonas Species

Extra-chromosomal factors of different molecular weight were found in all the 17 strains. It is possible to hypothesize that plasmid bands with high molecular weight could be responsible for the transferability of the biological characteristics and antibiotic resistance found in the present investigation. In fifteen strains, the plasmids with different molecular weights were present as follows: one large plasmid with different size from 48 to 52 kb and other plasmids with low molecular weight ranging from 3.7 to 1.7 kb. Moreover, in two strains, only plasmids with low molecular weight (1.8 Kb) were present. Examples of some pseudomonas plasmid profiles are shown in Fig. 2.

Biofilm Formation by the Six Selected Strains

All six selected *Pseudomonas* strains were biofilm producers after 72 h at 30 °C, but with different growth kinetics. *P. aeruginosa* 98.5*, P. fluorescens* 97.4 showed a good adhesion capability, reaching a bacterial load of 10^7 CFU/cm^2. This capability remained almost unchanged throughout the experiment and was expressed for both the polystyrene and glass surfaces employed. Intermediate biofilm forming attitude was expressed by the remaining *Pseudomonas* strains (Fig. 3).

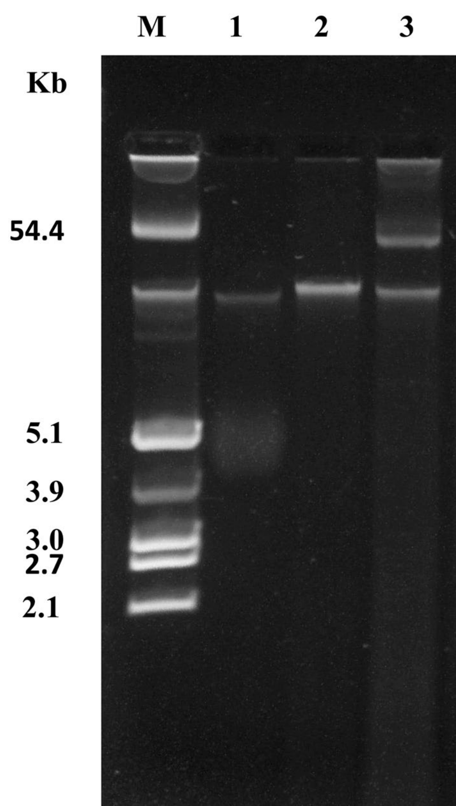

Fig. 2 Examples of some pseudomonas plasmid profiles. Lane M: molecular size markers prepared from *E. coli* V517 (54.4, 5.6, 5.1, 3.9, 3.0, 2.7 and 2.1 kb); lane 1: *P. aeruginosa* 95.1; lane 2: *P. fluorescens* 97.4; lane 3: *P. putida 96.3*

Discussion

The aquatic ecosystem represents one of the most interesting environments to study microbial ecology. These are in fact colonized by numerous Gram-negative species, mainly belonging to the genera *Pseudomonas*, that for many years have attracted the attention of researchers due to their peculiar biological characteristics. The variation in virulence profiles, reflecting the adaptive capability of *Pseudomonas* strains, highlights their attitude to hypercolonize aquatic environments. The production of bacteriocins, for example, is an important feature that confers an ecological advantage to the producing strains, compared to other microorganisms that harbor the same ecosystem, and can contribute to the colonization or invasion of an ecological niche. Adhesion and motility are closely linked characteristics and, therefore, it can be deduced that many of the pseudomonas isolates are endowed with a high ability to adhere to surfaces commonly found in the health care environments. The motility is an important feature influencing biofilm formation and, as demonstrated by Overhage et al. [32], many mutants with altered swarming motility were also defective in biofilm formation. Of recent interest are the soluble virulence factors (hemolysin, gelatinase, DNase, urease and siderophore production) secreted by some *Pseudomonas* strains, involved both in the interactions between bacteria that share the same habitat and human infections. Hemolysin is a pore-forming toxin able to cause the destruction of cells by lysis. Another notable virulence factor, gelatinase, allows the bacteria to hydrolyze gelatin (a protein found in connective tissue) and to metabolize the small peptides that originate from its hydrolysis for energy. The gelatinase activity, often observed in this study, could strengthen their virulence, making them potentially pathogenic, especially for immunocompromised subjects. DNase is a useful virulence factor for bacteria, helping them escape viscous secretions and NETs (neutrophil extracellular traps), which are chromatin formations involved in trapping and killing *P. aeruginosa* during respiratory infections [33]. The role of urease in the virulence of *P. aeruginosa* is well established [34]. It is essential for the colonization of a host organism and, due to its enzymatic activity, it has a toxic effect on human cells. Ureolytic activity is often observed in many pathogens, and it is recognized as one of the major bacterial virulence factors during urinary tract infections caused by the producing strains [35]. Lastly, the production of siderophores, frequently involved in chronic infections,

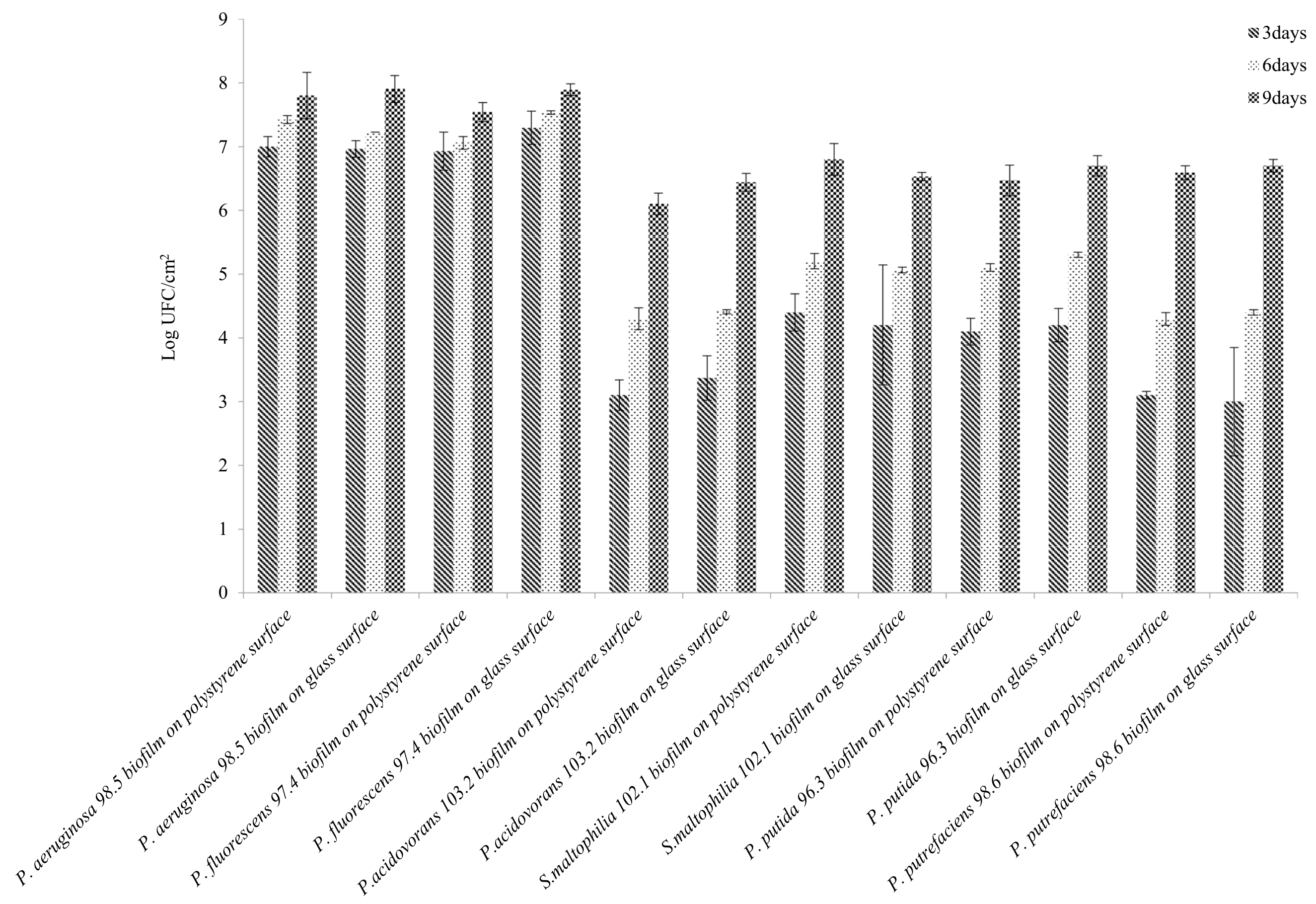

Fig. 3 Biofilms produced by the species *P. aeruginosa* 98.5, *P. fluorescens* 97.4, *P. acidovorans* 103.2, *S. maltophilia* 102.1, *P. putida* 96.3, *and P. putrefaciens* 98.6 on 12-well polystyrene microtiter plates and glass coupons

enables the bacteria to multiplicate and growth when no or low iron is available [36].

The simultaneous presence of some important biological characteristics and the ability to form biofilms on different surfaces can be a further reason to confirm the virulence of these hydrophilic microorganisms. Motility, biofilm formation, and host invasion are in fact some of the expressions of functional responses to surface colonization.

Although our study was conducted on a limited sample of bacteria, the results obtained still provide some important information. One major and recognized characteristic that makes bacteria potential pathogens for human is the possession of virulence factors. *P. aeruginosa* confirms not only its virulence attitude, but also the capability for environmental competition. This pathogen has shown to possess almost all virulence factors, including those responsible for environmental competition, such as the production of antibacterial substances and siderophore, motility, and biofilm formation, the last two mainly involved in the pathogenicity of nosocomial strains. Similarly, *P. fluorescens* 97.4 was positive for most tests, in particular for all three forms of mobility and biofilm formation, features indispensable both for the pathogenicity, and for the colonization of an ecological niche. The capacity for environmental competition in *P. acidovorans*, evidenced by the production of substances with antibacterial activity, is probably the main factor behind its greater presence in the water sector, as can be seen from our study where it is the second frequently isolated species. Lastly, most of our isolates also showed one or more antibiotic resistance. The spreading of multidrug-resistant bacteria through hospital wastewater is a cause of concern, especially for hydrophilic species, often organized in biofilms, for which the hospital wastewater environment may represent an ideal habitat. From a biological point of view, the development of resistance phenotypes could be related to bacterial survival and adaptation processes, as a response to the selective pressure present in nosocomial environments. Resistance of *P. aeruginosa* to antibiotics is a major problem, and the potential of hospital waste systems to act as a reservoir for MDR *P. aeruginosa* and other nosocomial pathogens was pointed out by Breathnach et al. [37]. Targeting virulence

factors, in addition to pathogenic behaviors such as swimming, swarming, and twitching motilities and biofilm formation, is an alternative option to control the emergence of resistance to antibiotics [38]. The simultaneous presence of multidrug resistance and the capability to form biofilm on different surfaces can be considered a further demonstration of the potential of these bacteria to colonize every type of substrate, including drinking water distribution systems (DWDS) and medical devices, and the presence of these microorganisms would make it difficult to employ effective therapies or adequate disinfection strategies. Anaissie et al. [39] pointed out that hospital water distribution systems might be the most overlooked, important, and controllable source of HAIs. In a longitudinal study conducted by Vallés et al. [40] in an intensive care unit over a 3-year period, it was shown that 54 percent of ventilated patients were colonized with *P. aeruginosa* and about half of these were isolated from tap water. Similarly, Blanc et al. [41], performing a study on 5 ICUs in Lausanne, Switzerland, found a strain identical to one isolated from water faucets in 42% of their patients. Association between healthcare water systems and *P. aeruginosa* infections was also reported by Loveday et al. [42].

Waters in general, and tap water in particular, are often colonized by many *Pseudomonas* species. The current Italian legislation does not require their determination in water system, except for *P. aeruginosa*. Given that the phenomenon of bacterial growth in water is widely recognized, the detection and knowledge of the biological characteristics responsible for the pathogenic attitude of *Pseudomonas* strains other than *P. aeruginosa* would be of a certain importance to prevent infectious diseases in hospital settings. The present investigation has highlighted the presence of some biological characteristics, such as the gelatinase activity, hydrolysis of casein, and so on, observed in a high percentage of isolates, which could enhance the virulence of these bacteria, making them potentially pathogenic, especially against immunocompromised patients. It would therefore be important to perform routine monitoring of water used in medical environments, not only related to *P. aeruginosa*, but also related to other *Pseudomonas* species that, as emerged in this study, possess numerous virulence characteristics that could increase the risk of waterborne-transmitted diseases, especially in the healthcare setting. For this reason, deep knowledge and understanding on how different virulence factors are involved in the development of infectious diseases by species until now considered non-pathogenic for humans, could play a significant role, thus contributing to preventive procedures able to reduce HAI caused by waterborne microorganisms.

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Compliance with Ethical Standards

Conflict of interest The author reports no conflict of interest in this work.

References

1. Carpenter RJ, Hartzell JD, Forsberg JA, Babel BS, Ganesan A (2008) *Pseudomonas putida* war wound infection in a US marine: a case report and review of the literature. J Infect 56:234–240
2. Noble RC, Overman SB (1994) *Pseudomonas stutzeri* infection. A review of hospital isolates and a review of the literature. Diagn Microbiol Infect Dis 19:51–56
3. Picot L, Abdelmoula SM, Merieau A, Leroux P, Cazin L, Orange N, Feuilloley MG (2001) *Pseudomonas fluorescens* as a potential pathogen: adherence to nerve cells. Microb Infect 3:985–995
4. Scales BS, Dickson RP, LiPuma JJ, Huffnagle Gary B (2014) Microbiology, Genomics, and clinical significance of the *Pseudomonas fluorescens* species complex, an unappreciated colonizer of humans. Clin Microbiol Rev 27:927–948
5. Kim SE, Park SH, Park HB, Park KH, Kim SH, Jung SI, Shin JH, Jang HC, Kang SJ (2012) Nosocomial *Pseudomonas putida* bacteremia: high rates of carbapenem resistance and mortality. Chonnam Med J 48:91–95
6. Erol S, Zenciroglu A, Dilli D, Okumus N, Aydin M, Gol N, Erdem F, Tanir G (2014) Evaluation of nosocomial blood stream infections caused by *Pseudomonas* species in newborns. Clin Lab 460:615–620
7. Yoshino Y, Kitazawa T, Kamimura M, Tatsuno K, Ota Y, Yotsuyanagi H (2011) *Pseudomonas putida* bacteremia in adult patients: five case reports and a review of the literature. J Infect Chemother 17:278–282
8. Fernández M, Porcel M, de la Torre J, Molina-Henares MA, Daddaoua A, Llamas MA, Roca A, Carriel V, Garzón I, Ramos JL, Alaminos M, Duque E (2015) Analysis of the pathogenic potential of nosocomial *Pseudomonas putida* strains. Front Microbiol 6:871
9. Huang CR, Lien CY, Tsai NW, Lai WA, Hsu CW, Tsai NW, Chang CC, Lu CH, Chien CC, Chang WN (2018) The clinical characteristics of adult bacterial meningitis caused by non-*Pseudomonas* (*Ps.*) *Pseudomonas* species: a clinical comparison with *Ps. aeruginosa meningitis*. Kaohsiung J Med Sci 34:49–55
10. Prakash B, Veeregowda BM, Krishnappa G (2003) Biofilms: a survival strategy of bacteria. Curr Sci 85:1299–1307
11. Messi P (2013) Biofilm formation, development and relevance. In: Simões M, Mergulhão F (eds) Biofilm in bioengineering. Nova Science, Hauppauge, p 268
12. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial biofilms. Ann Rev Microbiol 49:711–745
13. Davey ME, Otoole GA (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 64:847–867
14. Garrett TR, Bhakoo M, Zhang Z (2008) Bacterial adhesion and biofilms on surfaces. Proc Natl Sci 18:1049–1056
15. Lewandowski Z, Beyenal H (2014) Fundamentals of biofilm research, 2nd edn. Taylor & Francis, Boca Raton

16. Molina L, Udaondo Z, Duque E, Fernàndez M, Molina-Santiago C, Roca A, Porcel M, de la Torre J, Segura A, Plesiat P, Jeannot K, Ramos JL (2014) Antibiotic resistance determinants in a *Pseudomonas putida* strain isolated from a hospital. PLoS ONE 9:e81604. https://doi.org/10.1371/journal.pone.0081604
17. Martino R, Martínez C, Pericas R, Salazar R, Solá C, Brunet S, Sureda A, Domingo-Albos A (1996) Bacteremia due to glucose non-fermenting gram-negative bacilli in patients with hematological neoplasias and solid tumors. Eur J Clin Microbiol Infect Dis 15:610–615
18. Yoshino Y, Kitazawa T, Kamimura M, Tatsuno K, Ota Y, Yotsuyanagi H (2011) *Pseudomonas putida* bacteremia in adult patients: five case reports and a review of the literature. J Infect Chemotherm 17:278–282
19. Aumeran C, Paillard C, Robin F, Kanold J, Baud O, Bonnet R, Souweine B, Traore O (2007) *Pseudomonas aeruginosa* and *Pseudomonas putida* outbreak associated with contaminated water outlets in an oncohaematology paediatric unit. J Hosp Infect 65:47–53
20. Moreira IV, Nunes OC, Manaia CM (2012) Diversity and antibiotic resistance in *Pseudomonas* spp. from drinking water. Sci Tot Environ 426:366–374
21. UNI EN ISO 16266 (2008) Water quality. Detection and enumeration of *Pseudomonas aeruginosa*. Method by membrane filtration. International Organization for Standardization, Technical committee ISO/TC 147, Subcommittee SC 4
22. Alm EW, Oerther DB, Larsen N, Sthal DA, Raskin L (1996) The oligonucleotide probe database. Appl Environ Microbiol 62:3557–3559
23. NCBI National Center for Biotechnology Information (2009) BLAST, a basic local alignment search tool. https://blast.ncbi.nlm.nih.gov
24. Schwyn R, Neilands JB (1987) Universal chemical assay for detection and determination of siderophores. Anal Biochem 160:47–56
25. Kohler T, Curty LK, Barja F, van Delden C, Pechere JC (2000) Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J Bacteriol 182:5990–5996
26. Rashid MH, Kornberg A (2000) Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of Pseudomonas aeruginosa. Proc Natl Acad Sci USA 97:4885–4890
27. Fonseca AP, Correia P, Sousa JC, Tenreiro R (2007) Association patterns of *Pseudomonas aeruginosa* clinical isolates as revealed by virulence traits, antibiotic resistance, serotype and genotype. FEMS Immunol Med Microbiol 51:505–516
28. Kekessy DA, Piquet JD (1970) New method for detecting bacteriocin production. Appl Microbiol 20:282–283
29. CLSI 2018 (Clinical and Laboratory Standard Institutes) (2018) Performance standards for antimicrobial susceptibility testing. CLSI supplement M100, 28th edn. Clinical and Laboratory Standard Institutes, Wayne
30. European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2018) Clinical-breakpoints. Version 8.0, valid from 2018-01. https://www.eucast.org/clinical_breakpoints/.
31. Macrina FL, Kopecko DJ, Jones KR, Ayers DJ, McCowen SM (1978) A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid 1:417–420
32. Overhage J, Lewenza S, Marr AK, Hancock REW (2007) Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 mini-Tn5-lux mutant library. J Bacteriol 189:2164–2169
33. Anghel I, Holban AM, Grumezescu AM, Andronescu E, Ficai A, Anghel G, Maganu M, Lazar V, Chifiriuc MC (2012) Modified wound dressing with phyto-nanostructured coating to prevent staphylococcal and pseudomonal biofilm development. Nanoscale Res Lett 7:690
34. Bradbury RS, Reid DW, Champion AC (2014) Urease production as a marker of virulence in *Pseudomonas aeruginosa*. Brit J Biomed Sci 71:175–177
35. Mobley HL, Island MD, Hausinger RP (1995) Molecular biology of microbial ureases. Microbiol Rev 59:451–480
36. Khalifa BHA, Moissenet D, Vu TH, Khedher M (2011) Virulence factors in *Pseudomonas aeruginosa*: mechanisms and modes of regulation. Ann Biol Clin 69:393–403
37. Breathnach AS, Cubbon MD, Karunaharan RN, Pope CF, Planche TD (2012) Multidrug-resistant *Pseudomonas aeruginosa* outbreaks in two hospitals: association with contaminated hospital waste-water systems. J Hosp Inf 82:19–24
38. Abbas HA (2015) Inhibition of virulence factors of *Pseudomonas aeruginosa* by diclofenac sodium. Roum Arch Microbiol Immunol 74:79–85
39. Anaissie E, Penzak S, Dignani C (2002) The hospital water supply as a source of nosocomial infections. Arch Intern Med 162:1483–1492
40. Vallés J, Mariscal D, Cortes P, Coll P, Villagra A, Diaz E, Artigas A, Rello J (2004) Patterns of colonization by *Pseudomonas aeruginosa* in intubated patients: a 3-year prospective study of 1,607 isolates using pulsed-field gel electrophoresis with implications for prevention of ventilator-associated pneumonia. Intensive Care Med 30:1768–1775
41. Blanc DS, Nahimana I, Petignat C, Wenger A, Bille J, Francioli P (2004) Faucets as a reservoir of endemic *Pseudomonas aeruginosa* colonization/infections in intensive care units. Intensive Care Med 30:1964–1968
42. Loveday HP, Wilson JA, Kerr K, Pitchers R, Walker JT, Browne J (2014) Association between healthcare water systems and *Pseudomonas aeruginosa* infections: a rapid systematic review. J Hosp Infect 86:7–15

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