

Article

Phenotypical and Molecular Characterization of *Pseudomonas* spp. Isolated from a Pharmaceutical Facility

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Abstract

The characterization of environmental *Pseudomonas* spp. from pharmaceutical industries is a relatively underexplored area of research. This study used 40 isolates of *Pseudomonas* from a pharmaceutical company that had been presumptively identified as *Pseudomonas aeruginosa* by VITEK[®]2. The isolates were characterized using 16S rRNA sequencing, Multilocus Sequence Typing (MLST), Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass (MALDI–TOF MS), Fourier Transform Infrared (FT–IR) spectroscopy, ERIC–PCR, antimicrobial susceptibility profiling, and biofilm formation assessment on stainless steel surfaces. Twenty isolates were confirmed as *P. aeruginosa*. Sixteen isolates were only identified to the genus level of *Pseudomonas*, and the remaining four isolates were identified as *Enterococcus faecalis* ($n = 2$), *Proteus* spp. ($n = 1$), and *Staphylococcus saprophyticus* subsp. *bovis*/*S. edaphicus*/*S. saprophyticus* subsp. *saprophyticus*/*S. pseudoxylus*/*S. xylosus*/*S. caeli*/*S. ureilyticus*. Typing of the 20 *P. aeruginosa* strains yielded 18 distinct FT–IR profiles and 19 ERIC–PCR profiles. The MLST analysis identified eight new sequence types (ST4292–ST4299). All strains were classified as multidrug-resistant (MDR), exhibiting resistance to multiple antimicrobial classes. Biofilm formation was observed in 28 (77.8%) *Pseudomonas* spp. strains on polystyrene surfaces, classified as moderately or strongly adherent, while all *P. aeruginosa* strains formed biofilms on stainless steel. Of the three disinfectants tested, sodium hypochlorite at 0.01% showed the best performance, reducing or eliminating biofilm formation in 24 (85.7%) strains. The regular evaluation of disinfection effectiveness in pharmaceutical industries is essential, as the presence of biofilm-forming strains can compromise production and contaminate final products.

Keywords: *Pseudomonas aeruginosa*; biofilm; MLST; MALDI–TOF MS; FT–IR; biopharmaceutical facility

1. Introduction

Pseudomonas aeruginosa is a bacterium that is well recognized for its clinical relevance [1] and is commonly found in the water (potable, purified, ultrapurified, for injectables) used in pharmaceutical industries [2,3]. According to quality control guidelines



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applied for the pharmaceutical industries producing sterile products, such as vaccines and biologicals, *P. aeruginosa* are not permissible to be present in raw materials and/or the potable water used [4,5]. In addition, Mattoso et al. (2025) [3] proposed a criterion for the classification of microorganisms identified in bioburden analysis, and that *P. aeruginosa* should be considered as a critical organism that results in a production lot being discarded.

The ability of *P. aeruginosa* to form biofilms reinforces why it is a pathogenic organism of concern as it can persist on abiotic surfaces, equipment, and in water piping systems [6]. In biofilm communities, the extracellular matrix increases bacterial tolerance to disinfectants and antibiotic agents, contributing to biofilm persistence and the contamination of pharmaceutical industrial environments [7–10].

Additionally, *P. aeruginosa* is a pathogen commonly associated with healthcare-associated infections, causing severe infections, especially in immunocompromised patients, and can show resistance to multiple antimicrobials [11–13]. Antimicrobial resistance (AMR) is a serious and growing global health threat. Globally, increasing antibiotic resistance trends in Gram-negative bacterial pathogens pose a growing threat [14]. In 2024, the World Health Organization (WHO) updated the Bacterial Priority Pathogens List, which covers bacterial pathogens of public health importance to guide research, development, and strategies to prevent and control antimicrobial resistance. Subsequently, carbapenem-resistant *P. aeruginosa* was categorized as an organism of high priority [15]. The monitoring of this pathogen in clinical and non-clinical environments is important for a One Health approach to understand its epidemiology and to control its dissemination [16–20]. Consequently, phenotypical and molecular studies of *P. aeruginosa* strains isolated from pharmaceutical industry facilities are important for quality control and the safety of pharmaceutical products [21], as well as improving our understanding of the diversity of strains in the production environment. Although some studies have focused on the clinical and environmental relevance of *P. aeruginosa*, a comprehensive polyphasic characterization of strains from biopharmaceutical facilities remains underexplored.

The objective of this study was to characterize by phenotypical and molecular techniques *P. aeruginosa* strains isolated from a pharmaceutical industry facility, and to evaluate the antimicrobial susceptibility profile and biofilm production of the isolates.

2. Materials and Methods

2.1. Bacterial Isolates and Culture Conditions

Forty isolates obtained from an immunobiological pharmaceutical facility, located in Rio de Janeiro State, Brazil, between 2015 and 2020 were analyzed. They were initially identified as *P. aeruginosa* by VITEK®2 systems 07.01 (bioMérieux, Craponne, France). They had been isolated from different sources: water ($n = 21$), sterility tests ($n = 13$), bioburden tests ($n = 4$), cleaning validation ($n = 1$), and active pharmaceutical ingredient (API) ($n = 1$) (Table 1). The strains were then deposited at the Coleção de Bactérias do Ambiente e Saúde (CBAS) culture collection hosted at Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil (www.cbas.fiocruz.br (accessed on 5 January 2026)). CBAS is affiliated with the World Federation for Culture Collections (WFCC).

Table 1. Molecular characterization of the isolates previously identified as *P. aeruginosa* by VITEK®2 system ($n = 40$) in a pharmaceutical industry facility.

Strain	Source	Year	MALDI Biotyper® (Score)	VITEK® MS (%)	Full Gene 16S rRNA Sequencing—Access Number	ST ^a
B049/15	Sterility test	2015	<i>P. aeruginosa</i> (2.47)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613268	4298
B144/15	Bioburden test	2015	<i>P. koreensis</i> (2.05)	<i>Pseudomonas</i> spp. (77.3)	<i>P. koreensis</i> / <i>P. iranensis</i> / <i>P. atacamensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomasticsae</i> / <i>P. jessenii</i> / <i>P. moraviensis</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. siliginis</i> / <i>P. triticicola</i> / <i>P. baetica</i> / <i>P. granadensis</i> / <i>P. moorei</i> / <i>P. vancouverensis</i> / <i>P. laurylsulfativorans</i> / <i>P. khorasanensis</i> / <i>P. izuensis</i> / <i>P. mونسensis</i> / <i>P. hamedanensis</i> / <i>P. neuropathica</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. fulva</i> / <i>P. helmanticensis</i> / <i>P. rustica</i> / <i>P. zeae</i> —PQ464611	NR ^b
B265/15	Water	2015	<i>P. koreensis</i> (2.03)	<i>Pseudomonas</i> spp. (87.8)	<i>P. koreensis</i> / <i>P. iranensis</i> / <i>P. atacamensis</i> / <i>P. gozinkensis</i> / <i>P. fitomasticsae</i> / <i>P. reinekei</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. jessenii</i> / <i>P. moraviensis</i> / <i>P. granadensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. triticicola</i> / <i>P. baetica</i> / <i>P. vancouverensis</i> / <i>P. moorei</i> / <i>P. izuensis</i> / <i>P. siliginis</i> / <i>P. khorasanensis</i> / <i>P. laurylsulfativorans</i> / <i>P. mونسensis</i> / <i>P. hamedanensis</i> / <i>P. neuropathica</i> / <i>P. atagonensis</i> / <i>P. tensinigenes</i> / <i>P. fulva</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. parafulva</i> —PQ464612	NR
B527/15	Water	2015	<i>P. aeruginosa</i> (2.15)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613251	640
B764/15	Water	2015	<i>P. aeruginosa</i> (2.03)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613252	4292
B1815/15	Water	2015	<i>P. koreensis</i> (2.04)	NI ^c	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613253	ND ^d
B056/16	Sterility test	2016	<i>P. aeruginosa</i> (2.16)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613254	213
B380/16	Cleaning validation	2016	<i>P. aeruginosa</i> (2.33)	<i>P. aeruginosa</i> (99.0)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613255	ND
B546/16	Water	2016	<i>P. aeruginosa</i> (2.28)	<i>P. aeruginosa</i> (86.1)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613256	4293
B825/16	Sterility test	2016	<i>P. aeruginosa</i> (2.03)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613257	308
B406/17	Water	2017	NR	NR	<i>Staphylococcus saprophyticus</i> subsp. <i>bovis</i> / <i>S. edaphicus</i> / <i>S. saprophyticus</i> subsp. <i>saprophyticus</i> / <i>S. pseudoxylus</i> / <i>S. xylosus</i> / <i>S. caeli</i> / <i>S. ureilyticus</i> —PQ464613	NR
B426/17	Sterility test	2017	<i>P. aeruginosa</i> (2.05)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613258	17
B587/17	Sterility test	2017	<i>P. aeruginosa</i> (2.33)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613259	2289
B596/17	Water	2017	NR	NR	<i>Enterococcus faecalis</i> —PQ464614	NR
B718/17	Bioburden test	2017	NR	NR	<i>Enterococcus faecalis</i> —PQ497692	NR
B161/18	Sterility test	2018	<i>P. aeruginosa</i> (2.30)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613260	4294

Table 1. Cont.

Strain	Source	Year	MALDI Biotyper [®] (Score)	VITEK [®] MS (%)	Full Gene 16S rRNA Sequencing—Access Number	ST ^a
B180/18	Water	2018	<i>P. koreensis</i> (2.37)	<i>Pseudomonas</i> spp. (86.2)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaticsae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. moraviensis</i> / <i>P. granadensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. siliginis</i> / <i>P. vancouverensis</i> / <i>P. triticicola</i> / <i>P. moorei</i> / <i>P. izuensis</i> / <i>P. baetica</i> / <i>P. laurylsulfativorans</i> / <i>P. monsensis</i> / <i>P. khorasanensis</i> / <i>P. atagonensis</i> / <i>P. hamedanensis</i> / <i>P. fulva</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. neuropathica</i> / <i>P. parafulva</i> / <i>P. helmanticensis</i> / <i>P. rustica</i> / <i>P. zeae</i> / <i>P. umsongensis</i> —PQ497968	NR
B188/18	Sterility test	2018	<i>P. aeruginosa</i> (2.37)	<i>P. aeruginosa</i> (95.6)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613261	4295
B216/18	Sterility test	2018	<i>P. aeruginosa</i> (2.18)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613262	2287
B284/18	Sterility test	2018	<i>P. aeruginosa</i> (2.39)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613263	2963
B576/18	Sterility test	2018	NR	NR	<i>Proteus</i> spp.—PQ497784	NR
B877/18	Water	2018	<i>P. koreensis</i> (1.99)	<i>Pseudomonas</i> spp. (94.8)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. reinekei</i> / <i>P. iranensis</i> / <i>P. gessardii</i> / <i>P. gozinkensis</i> / <i>P. glycinae</i> / <i>P. fitomaticsae</i> / <i>P. synxantha</i> / <i>P. migulae</i> / <i>P. jessenii</i> / <i>P. vancouverensis</i> / <i>P. libanensis</i> / <i>P. allokribbensis</i> / <i>P. shahriarae</i> / <i>P. moorei</i> / <i>P. izuensis</i> / <i>P. khavaziana</i> / <i>P. citri</i> / <i>P. mucidolens</i> / <i>P. cedrina</i> subsp. <i>cedrina</i> / <i>P. moraviensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. monsensis</i> / <i>P. paracarnis</i> —PQ464615	NR
B926/18	Water	2018	<i>P. aeruginosa</i> (2.07)	<i>Pseudomonas</i> spp. (94.8)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaticsae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. moraviensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. granadensis</i> / <i>P. siliginis</i> / <i>P. triticicola</i> / <i>P. vancouverensis</i> / <i>P. baetica</i> / <i>P. moorei</i> / <i>P. laurylsulfativorans</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. monsensis</i> / <i>P. hamedanensis</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. fulva</i> / <i>P. neuropathica</i> / <i>P. parafulva</i> / <i>P. helmanticensis</i> / <i>P. rustica</i> / <i>P. zeae</i> / <i>P. umsongensis</i> —PQ464616	NR
B1025/18	Water	2018	<i>P. aeruginosa</i> (2.29)	<i>P. aeruginosa</i> (99.2)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613264	4296
B1142/18	API ^e	2018	<i>P. koreensis</i> (1.65)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613265	2865
B1050/18	Water	2018	<i>P. aeruginosa</i> (2.04)	<i>P. aeruginosa</i> (95.4)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613266	2230

Table 1. Cont.

Strain	Source	Year	MALDI Biotyper [®] (Score)	VITEK [®] MS (%)	Full Gene 16S rRNA Sequencing—Access Number	ST ^a
B1151/18	Water	2018	<i>P. aeruginosa</i> (2.18)	<i>Pseudomonas</i> spp. (99.9)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaccae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glyciniae</i> / <i>P. moraviensis</i> / <i>P. azərbayjanoccidentalis</i> / <i>P. baetica</i> / <i>P. granadensis</i> / <i>P. siliginis</i> / <i>P. triticicola</i> / <i>P. vancouverensis</i> / <i>P. moorei</i> / <i>P. laurylsulfativorans</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. mونسensis</i> / <i>P. hamedanensis</i> / <i>P. neuropathica</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. fulva</i> —PQ464617	NR
B1224/18	Water	2018	<i>P. koreensis</i> (1.37)	<i>Pseudomonas</i> spp. (95.4)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaccae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glyciniae</i> / <i>P. moraviensis</i> / <i>P. azərbayjanoccidentalis</i> / <i>P. granadensis</i> / <i>P. siliginis</i> / <i>P. triticicola</i> / <i>P. vancouverensis</i> / <i>P. baetica</i> / <i>P. moorei</i> / <i>P. laurylsulfativorans</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. mونسensis</i> / <i>P. hamedanensis</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. neuropathica</i> / <i>P. parafulva</i> / <i>P. helmanticensis</i> / <i>P. rustica</i> / <i>P. zeae</i> —PQ464618	NR
B1675/18	Water	2018	<i>P. koreensis</i> (1.97)	NI	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaccae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glyciniae</i> / <i>P. moraviensis</i> / <i>P. azərbayjanoccidentalis</i> / <i>P. granadensis</i> / <i>P. siliginis</i> / <i>P. triticicola</i> / <i>P. vancouverensis</i> / <i>P. baetica</i> / <i>P. moorei</i> / <i>P. laurylsulfativorans</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. mونسensis</i> / <i>P. hamedanensis</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. neuropathica</i> / <i>P. parafulva</i> / <i>P. helmanticensis</i> / <i>P. rustica</i> / <i>P. zeae</i> / <i>P. umsongensis</i> —PQ464619	NR
B1761/18	Water	2018	<i>P. koreensis</i> (1.91)	<i>Pseudomonas</i> spp. (80.7)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaccae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glyciniae</i> / <i>P. moraviensis</i> / <i>P. azərbayjanoccidentalis</i> / <i>P. granadensis</i> / <i>P. siliginis</i> / <i>P. triticicola</i> / <i>P. vancouverensis</i> / <i>P. baetica</i> / <i>P. moorei</i> / <i>P. laurylsulfativorans</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. mونسensis</i> / <i>P. hamedanensis</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. neuropathica</i> / <i>P. parafulva</i> / <i>P. helmanticensis</i> / <i>P. rustica</i> / <i>P. zeae</i> —PQ464620	NR

Table 1. Cont.

Strain	Source	Year	MALDI Biotyper [®] (Score)	VITEK [®] MS (%)	Full Gene 16S rRNA Sequencing—Access Number	ST ^a
B1888/18	Water	2018	<i>P. koreensis</i> (1.94)	<i>Pseudomonas</i> spp. (95.6)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaticsae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. moraviensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. granadensis</i> / <i>P. siliginis</i> / <i>P. triticicola</i> / <i>P. vancouverensis</i> / <i>P. baetica</i> / <i>P. moorei</i> / <i>P. laurylsulfativorans</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. monsensis</i> / <i>P. hamedanensis</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. neuropathica</i> / <i>P. parafulva</i> —PQ464621	NR
B1905/18	Water	2018	<i>P. koreensis</i> (1.83)	<i>Pseudomonas</i> spp. (95.6)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaticsae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. moraviensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. granadensis</i> / <i>P. siliginis</i> / <i>P. triticicola</i> / <i>P. vancouverensis</i> / <i>P. baetica</i> / <i>P. moorei</i> / <i>P. laurylsulfativorans</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. monsensis</i> / <i>P. hamedanensis</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. neuropathica</i> / <i>P. parafulva</i> / <i>P. helmanticensis</i> / <i>P. rustica</i> / <i>P. zeae</i> —PQ464622	NR
B1934/18	Water	2018	<i>P. koreensis</i> (1.86)	NI	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaticsae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. moraviensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. granadensis</i> / <i>P. siliginis</i> / <i>P. triticicola</i> / <i>P. vancouverensis</i> / <i>P. baetica</i> / <i>P. moorei</i> / <i>P. laurylsulfativorans</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. monsensis</i> / <i>P. hamedanensis</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. neuropathica</i> / <i>P. parafulva</i> / <i>P. helmanticensis</i> / <i>P. rustica</i> / <i>P. zeae</i> —PQ464623	NR
B042/19	Sterility test	2019	<i>P. aeruginosa</i> (2.32)	<i>P. aeruginosa</i> (99.0)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613267	4297
B048/19	Water	2019	<i>P. koreensis</i> (2.09)	<i>Pseudomonas</i> spp. (82.6)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaticsae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. moraviensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. baetica</i> / <i>P. granadensis</i> / <i>P. siliginis</i> / <i>P. triticicola</i> / <i>P. vancouverensis</i> / <i>P. moorei</i> / <i>P. laurylsulfativorans</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. monsensis</i> / <i>P. hamedanensis</i> / <i>P. neuropathica</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. parafulva</i> —PQ464624	NR

Table 1. Cont.

Strain	Source	Year	MALDI Biotyper [®] (Score)	VITEK [®] MS (%)	Full Gene 16S rRNA Sequencing—Access Number	ST ^a
B274/19	Water	2019	<i>P. koreensis</i> (2.09)	<i>Pseudomonas</i> spp. (77.3)	<i>P. koreensis</i> / <i>P. moraviensis</i> / <i>P. iranensis</i> / <i>P. atacamensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaticsae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. moraviensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. siliginis</i> / <i>P. siliginis</i> / <i>P. trititicola</i> / <i>P. granadensis</i> / <i>P. vancouverensis</i> / <i>P. laurylsulfativorans</i> / <i>P. moorei</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. monsensis</i> / <i>P. hamedanensis</i> / <i>P. neuropathica</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. helmanticensis</i> / <i>P. rustica</i> / <i>P. zeae</i> / <i>P. iridis</i> / <i>P. anatoliensis</i> / <i>P. parafulva</i> —PQ464625	NR
B084/20	Bioburden test	2020	<i>P. koreensis</i> (2.01)	<i>Pseudomonas</i> spp. (85.1)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaticsae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. moraviensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. siliginis</i> / <i>P. siliginis</i> / <i>P. trititicola</i> / <i>P. granadensis</i> / <i>P. vancouverensis</i> / <i>P. laurylsulfativorans</i> / <i>P. moorei</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. monsensis</i> / <i>P. hamedanensis</i> / <i>P. neuropathica</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. parafulva</i> / <i>P. rustica</i> / <i>P. zeae</i> / <i>P. umsongensis</i> —PQ464626	NR
B167/20	Bioburden test	2020	<i>P. koreensis</i> (1.97)	<i>Pseudomonas</i> spp. (92.4)	<i>P. koreensis</i> / <i>P. atacamensis</i> / <i>P. iranensis</i> / <i>P. gozinkensis</i> / <i>P. reinekei</i> / <i>P. fitomaticsae</i> / <i>P. jessenii</i> / <i>P. allokribbensis</i> / <i>P. glycinae</i> / <i>P. moraviensis</i> / <i>P. azerbaijanoccidentalis</i> / <i>P. siliginis</i> / <i>P. siliginis</i> / <i>P. trititicola</i> / <i>P. granadensis</i> / <i>P. vancouverensis</i> / <i>P. laurylsulfativorans</i> / <i>P. moorei</i> / <i>P. izuensis</i> / <i>P. khorasanensis</i> / <i>P. monsensis</i> / <i>P. hamedanensis</i> / <i>P. neuropathica</i> / <i>P. atagonensis</i> / <i>P. crudilactis</i> / <i>P. germanica</i> / <i>P. tensinigenes</i> / <i>P. parafulva</i> / <i>P. rustica</i> / <i>P. zeae</i> / <i>P. umsongensis</i> —PQ464627	NR
B262/20	Sterility test	2020	<i>P. aeruginosa</i> (2.27)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613269	532
B576/20	Sterility test	2020	<i>P. aeruginosa</i> (2.15)	<i>P. aeruginosa</i> (99.9)	<i>P. aeruginosa</i> / <i>P. paraaeruginosa</i> —OQ613270	4299

^a—Sequence type; ^b—Not realized; ^c—Not identified (<75%); ^d—Not determined; ^e—Active pharmaceutical ingredient.

P. aeruginosa ATCC 9027 and *Escherichia coli* ATCC 8739 were used as controls in each specific test. Stock cultures were prepared and maintained at $-70\text{ }^{\circ}\text{C}$ in Difco™ Skim Milk 30% (BD Biosciences, Franklin Lakes, NJ, USA), containing 30% glycerol (Merck KGaA, Darmstadt, Germany). Inocula were prepared by transferring one loopful into 3 mL fresh brain–heart infusion broth (BHI) (Merck, Darmstadt, Germany) and incubating at $37 \pm 1\text{ }^{\circ}\text{C}/24\text{ h}$. For daily use, cultures were maintained at $5 \pm 3\text{ }^{\circ}\text{C}$ on Tryptic Soy Agar (TSA) (BioCen do Brasil, Santa Cruz do Rio Pardo, Brazil).

2.2. Phenotypical Characterization by VITEK® 2

The profiles resulting from the 47 biochemical tests of GN VITEK®2 card were summarized and categorized in sequential Roman alphabet letters. The phenotypic diversity among isolates was assessed using Simpson's index (SI) and its confidence intervals calculated using the Comparing Partitions online tool (<http://www.comparingpartitions.info/index.php?link=Tool> (accessed on 5 January 2026)) [22,23].

2.3. Identification by 16S rRNA Gene Sequencing

Identification of the isolates was performed using complete 16S rRNA gene sequencing with the MicroSEQ™ Full Gene 16S rDNA kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. The sequencing data were analyzed using the 3500 Series Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Sequence data were processed with DNA Star LaserGene SeqMan software version 7.0.0, and identification results were retrieved from the database at <https://www.ezbiocloud.net/> [24]—Database Update: 23 August 2023 (accessed on 5 January 2026). Only species identifications with a score $\geq 98.7\%$ were considered valid. All sequence data were submitted to <https://www.ncbi.nlm.nih.gov> (accessed on 5 January 2026) and their accession numbers are listed in Table 1. The sequences of the isolates identified as *Pseudomonas* spp. were used to construct a phylogenetic tree based on multiple alignments of concatenated sequences using the Neighbor-joining method based on the Kimura 2-parameter model with the software MEGA 11 [25].

2.4. Identification by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass (MALDI–TOF MS)

Two MALDI–TOF MS systems were evaluated: VITEK® MS and MALDI Biotyper®, according to the manufacturer's instructions. For analysis using VITEK® MS, the isolates were cultured on sheep blood agar (SBA) plates (BioCen do Brasil, Santa Cruz do Rio Pardo, Brazil) and incubated at $32.5 \pm 2.5\text{ }^{\circ}\text{C}/48\text{ h}$. After, a portion of each colony was transferred to a slide in triplicate, combined with $0.5\text{ }\mu\text{L}$ of 70% formic acid, and allowed to dry. Following this, $1\text{ }\mu\text{L}$ of alpha-cyano-4-hydroxycinnamic acid matrix solution (VITEK MS-CHCA, bioMérieux, Craponne, France) was added. *E. coli* ATCC 8739 was used as control. The slides were then analyzed using the VITEK® MS RUO system (bioMérieux, Craponne, France) and the results were processed with SARAMIS Premium software version 4.0.0.14. Isolates showing identification results with a percentage of $\geq 75\%$ were deemed identified.

For analysis using the MALDI Biotyper® (Bruker Daltonics, Bremen, Germany), the isolates were cultured on BSA and incubated at $32.5 \pm 2.5\text{ }^{\circ}\text{C}$ for 24–48 h. Subsequently, a portion of the colony was transferred to a well on a MALDI Biotyper® disposable plate. One microlitre of 70% formic acid was applied to the well, and after drying, $1\text{ }\mu\text{L}$ of the HCCA matrix was added. In one of the wells on the plate, $1\text{ }\mu\text{L}$ of the bacterial test standard (BTS) was added, and after drying, $1\text{ }\mu\text{L}$ of the HCCA matrix was also added to this well. The generated mass spectra were analyzed using the MBT Compass HT v. 5.1.100 program and compared with the libraries available on the equipment (MBT Compass Library Revision

K, 2022). Results were interpreted based on the obtained score as follows: 0.00–1.69—no microorganism identification possible; 1.70–1.99—low confidence in species identification; and 2.00–3.00—high confidence in species identification.

2.5. Identification Methodology Comparison

The sensitivity, specificity, and accuracy of VITEK[®]2, VITEK[®]MS, and MALDI Biotyper[®] to identify *P. aeruginosa* were determined according to Greenhalgh [26]. A polyphasic approach was applied to consider the true-positive results, considering the possibilities obtained by 16S rRNA sequencing and MALDI–TOF MS results as the “gold standard methods”.

2.6. Typing by Fourier Transform Infrared (FT-IR) Spectroscopy Analysis

FT-IR typing was performed using the IR Biotyper[®] equipment (Bruker, Billerica, MA, USA) following the manufacturer’s instructions. The strains were cultured on TSA and incubated for 48 h at 37 ± 2 °C. A loop containing a portion of bacterial growth was transferred to a 1.5 mL microtube containing metal cylinders and 50 µL of 70% ethanol. The tube was vigorously homogenized using a tube homogenizer, followed by the addition of 50 µL of distilled water. Next, 15 µL of the suspension were transferred to spots, in triplicate, on a specific 96-well plate for the equipment. Infrared test standard (IRTS) 1 and IRTS 2 controls were pre-prepared according to the manufacturer’s instructions and 10 µL were added per spot, in duplicate. One well was used as a blank, with no inoculum added. After filling all the wells, the plate was kept in room temperature until complete drying. Subsequently, it was inserted into the equipment for reading. For result analysis, a dendrogram was created with the raw data to cluster the separation spectrum. The cut-off value on the dendrogram was automatically calculated using OPUS v.7.5 software (Bruker Optics-Daltonics GmbH). The distance matrix based on the average Euclidean distance and linkage algorithm reflects the separation distances of all strains.

The FT-IR profile diversity among isolates was assessed using SI and its confidence intervals as previously described.

2.7. Typing by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

P. aeruginosa strains were subjected to the ERIC-PCR technique according to the protocol described by Versalovic, Koeuth, and Lupski [27], using the primer ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'). DNA was extracted from overnight BHI cultures using the Dneasy Blood & Tissue Kit (Qiagen[®], Germantown, MD, USA), following the manufacturer’s guidelines. The concentration of DNA was determined with a Qubit 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The amplification products were analyzed by gel electrophoresis on a 2% agarose gel, using GelRed[®] stain (Thermo Fisher Scientific, Waltham, MA USA), for 120 min at 60 V. The molecular weight marker used was the 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA). The gel was photographed and analyzed using the iBright[™] CL1500 imaging system (Thermo Fisher Scientific, USA). Band patterns were analyzed using BioNumerics version 8.1 software (Applied Maths, Kortrijk, Belgium). The dendrogram was constructed using the Dice index and the UPGMA method [28].

The genetic diversity profile among isolates typed by ERIC-PCR was assessed using SI and its confidence intervals as previously described.

2.8. Multilocus Sequence Typing (MLST)

DNA extraction was performed as previously described. The MLST gene set (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) was amplified using primers and PCR conditions according to protocol available at MLST *P. aeruginosa* database (<https://pubmlst>.

[org/organisms/pseudomonas-aeruginosa](https://pubmlst.org/organisms/pseudomonas-aeruginosa) (accessed on 5 January 2026). The nucleotide sequences of each gene were trimmed to the appropriate length and then queried to the PubMLST database. Allele numbers were assigned, and the sequence types (STs) were determined by tools available in the database. New alleles and STs were assigned by the *P. aeruginosa* PubMLST database curators.

The MLST profiles were clustered using BioNumerics 8.1 software (Applied Maths, Sint-Martens-Latem, Belgium), employing a categorical coefficient. Visualization of the data was performed with the minimum spanning tree (MST) tool, and the eBURST algorithm was used to identify clonal complexes (CCs), including single-locus variants (SLVs) and double-locus variants (DLVs) [29].

The genetic diversity profile among isolates typed by MLST was assessed using SI and its confidence intervals as previously described.

2.9. Antimicrobial Susceptibility Testing Methods

The antimicrobial susceptibility testing (AST) was performed for the strains identified as *Pseudomonas* spp. The minimum inhibitory concentration (MIC) was assessed using the AST Card in the VITEK®2 system, following the manufacturer's guidelines. *E. coli* ATCC 25922 served as the quality control strain. Results were interpreted based on the standards set by the Brazilian Committee on Antimicrobial Susceptibility Testing [30], which follow the European Committee on Antimicrobial Susceptibility Testing recommendations [31], that classify the strains as susceptible, "susceptible, increased exposure" (SIE), and resistant.

The strains were classified as multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR) according to the criteria proposed by Magiorakos et al. [11]. The authors characterize MDR as non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial classes, XDR as non-susceptible to ≥ 1 agent in all but ≤ 2 categories, and PDR as non-susceptible to all antimicrobial agents in all antimicrobial classes. For this analysis, the SIE results were considered as non-susceptible.

2.10. Biofilm Formation on Polystyrene Surfaces and Its Tolerance to Disinfectants

Biofilm formation was evaluated on polystyrene surfaces using 96-well plates (Falcon®, Somerset, NJ, USA) following the procedure described by Luz et al. [32]. The tests were conducted at two different temperatures: 22.5 ± 2.5 °C and 37.0 ± 2 °C for 48 h in BHI. Each strain was tested in triplicate across three separate experiments and *P. aeruginosa* ATCC 27853 was used as control. The classification of strains was based on the cut-off optical density (ODC) in comparison with the OD of the negative control (BHI only): non-adherent (−) $OD \leq ODC$, weak (+) $ODC < OD \leq 2 \times ODC$, moderate (++) $2 \times ODC < OD \leq 4 \times ODC$, strong (+++) $4 \times ODC < OD$. The final classification was determined by the median of the nine results.

Strains identified as moderately and strongly adherent were selected for evaluating their tolerance to disinfectants following the protocol described by Vasconcellos et al. [10]. The selected disinfectants and time of contact were selected following the recommendations of the Cleaning and Disinfection Program of the pharmaceutical facility where the strains were isolated, and included the following: 70% alcohol/15 min (Merck, Darmstadt, Germany), 0.1% sodium hypochlorite/15 min (Brasquímica, Belo Horizonte, MG, Brazil), and 0.05% quaternary ammonium/20 min (Chemitec, São Paulo, SP, Brazil). Additionally, for strains that remained moderately or strongly adherent after treatment with 0.05% quaternary ammonium, a higher concentration (0.08%) was also tested.

Differences in the degree of biofilm formation at each temperature (22.5 and 37.0 °C) and disinfectant were examined by Wilcoxon signed ranks test using software R Core Team v. 4.2.0 (Vienna, Austria). The *p*-values < 0.05 were considered significant.

2.11. Biofilm Formation on Stainless Steel Surfaces

Strains identified as *P. aeruginosa* through 16S rRNA sequencing were examined using scanning electron microscopy (SEM) on stainless steel surfaces following the procedure described by Vasconcellos et al. [10]. The strains were cultured in 15 mL of BHI and incubated at 37 ± 2 °C for 24 h with shaking at 150 rotations per minute. A sterile 6-well polystyrene plate (Corning®, Glendale, AZ, USA) was prepared with 4.0 mL of bacterial suspension in each well, along with a stainless steel disk (15 mm diameter), which had been sterilized previously by autoclaving at 121 °C/30 min. The plate was incubated at 37 ± 2 °C for 48 h. Following incubation, the wells were washed twice with 2.0 mL phosphate-buffered saline (PBS) (Gibco-BRL, Gaithersburg, MD, USA). The biofilm was then fixed for 1 h using 2.5% glutaraldehyde in 0.1 M cacodylate buffer. After fixation, the biofilm was washed three times with PBS for 5 min, post-fixed for 15 min in 1% osmium tetroxide (OsO₄) (Electron Microscopy Sciences, Hatfield, PA, USA), and washed again 3 times in PBS/5 min each. The samples were dehydrated through a graded ethanol series (7.5, 15, 30, 50, 70%, 90, and 100%) for 15 min at each concentration and critical point dried with CO₂ using a Critical Point Dryer machine (Leica-Microsystems, Wetzlar, Germany). Then, they were sputter-coated with a 15 nm gold layer and examined with a Jeol JSM 6390 scanning electron microscope (Tokyo, Japan).

3. Results

3.1. Phenotypical and Molecular Characterization of *Pseudomonas* spp.

After 16S rRNA gene sequencing, 36/40 isolates were identified in the genus *Pseudomonas*: 20 (50.0%) as *P. aeruginosa*/*P. paraaeruginosa*, and 16 (40.0%) as non-*Pseudomonas aeruginosa*. The latter were a mixture of 46 possible *Pseudomonas* species (Table 1). The remaining non-*Pseudomonas* isolates (4/40) were identified as *Enterococcus faecalis* (*n* = 2), *Proteus* spp. (*n* = 1), and *Staphylococcus saprophyticus* subsp. *bovis*/*S. edaphicus*/*S. saprophyticus* subsp. *saprophyticus*/*S. pseudoxylosum*/*S. xylosum*/*S. caeli*/*S. ureilyticus* (*n* = 1), as shown in Table 1.

The 36 *Pseudomonas* spp. strains were assigned to eighteen (I–XVIII) different phenotypes (Table 2), with phenotype ‘I’ being the most prevalent (17/40). The calculated SI was 0.808 (0.691–0.925).

After proteomic characterization of the 36 *Pseudomonas* spp. strains by MALDI Biotyper®, 20 isolates (55.6%) were identified as *P. aeruginosa* and 16 (44.4%) as *P. koreensis*. VITEK® MS identified 19 (52.8%) strains as *P. aeruginosa* with a confidence level ranging from 86.1 to 99.9%; 14 (38.9%) as *Pseudomonas* spp. with a confidence level ranging from 77.3 to 99.9%; and five (8.3%) could not be identified (Table 1).

The typing of the 20 *P. aeruginosa* strains obtained from FT-IR resulted in 18 different profiles and SI = 0.989 (0.972–1.000). Only two clusters were formed: FT-IR 1 with strains B262/20 and B1050/18, and FT-IR 6 with strains B188/18 and B042/19 (Figure 1).

After ERIC-PCR analysis, 19 different ERIC profiles were found. The only cluster was ERIC Profile 5 formed by B587/17 and B161/18 strains (Figure 2).

Table 2. Phenotypical characterization of *Pseudomonas* spp. strains ($n = 36$).

Strain	Phenotypic Profile	Biofilm Formation		TZP ^b	CAZ ^c	CPM ^d	IPM ^e	Antibiogram (MIC ^a)		CIP ^h	TIG ⁱ	CST ^j
		22.5 °C	37.0 °C					MEM ^f	AMK ^g			
B049/15	XVI	(++) ^k	(+) ^l	SIE ^m (=8)	SIE (=4)	SIE (=4)	SIE (=1)	S ⁿ (=1)	S (<=2)	SIE (<=0.25)	R ^o (<=0.5)	S (<=0.5)
B144/15	I	(+)	(+)	SIE (=8)	SIE (=8)	SIE (=1)	SIE (=1)	S (=0.5)	S (<=2)	SIE (<=0.25)	R (<=0.5)	S (<=0.5)
B265/15	IV	(+)	(+)	SIE (=8)	R (=16)	R (<=1)	SIE (=2)	S (=1)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B527/15	XV	(++)	(+++) ^p	SIE (=8)	SIE (=4)	SIE (=2)	SIE (=2)	S (<=0.25)	S (=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B764/15	XVI	(+++)	(+)	SIE (<=4)	SIE (<=1)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B1815/15	I	(++)	(++)	SIE (=8)	R (=16)	R (<=1)	SIE (=2)	S (=1)	S (<=2)	SIE (<=0.25)	R (<=0.5)	S (<=0.5)
B056/16	XIV	(+++)	(++)	SIE (=8)	SIE (=4)	SIE (=2)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B380/16	XVII	(+)	(++)	SIE (<=4)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B546/16	X	(+)	(++)	SIE (=8)	SIE (=4)	SIE (=2)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B825/16	XIV	(+++)	(+++)	SIE (<=4)	SIE (=4)	SIE (=2)	SIE (=1)	S (=0.5)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B426/17	XIV	(++)	(+)	SIE (<=4)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B587/17	XII	(++)	(++)	SIE (=8)	SIE (=4)	SIE (=2)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B161/18	XIV	(+++)	(++)	SIE (<=4)	SIE (=2)	SIE (=1)	SIE (=1)	S (=0.5)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B180/18	I	(+)	(+)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (<=0.5)	S (<=0.5)
B188/18	XV	(+++)	(+++)	SIE (<=4)	SIE (<=1)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B216/18	XIV	(+++)	(+)	SIE (<=4)	SIE (<=1)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B284/18	VI	(+)	(+)	SIE (=8)	SIE (=4)	SIE (=2)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B877/18	I	(++)	(+)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (<=0.5)	S (<=0.5)
B926/18	I	(++)	(+)	SIE (=8)	SIE (=2)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (<=0.5)	S (<=0.5)
B1025/18	IX	(+)	(+)	SIE (=8)	SIE (=2)	SIE (=1)	SIE (=2)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B1142/18	VIII	(+++)	(+++)	SIE (=8)	SIE (=2)	SIE (=1)	R (>=16)	SIE (=4)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B1050/18	VII	(++)	(++)	SIE (=8)	SIE (<=1)	SIE (=1)	SIE (=2)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B1151/18	I	(++)	(+)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)
B1224/18	I	(++)	(+)	SIE (<=4)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (=1)	S (<=0.5)
B1675/18	I	(++)	(+)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (<=0.5)	S (<=0.5)
B1761/18	I	(++)	(+)	SIE (=8)	SIE (<=1)	SIE (=1)	SIE (>=0.25)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (<=0.5)	S (<=0.5)
B1888/18	I	(++)	(+)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (<=0.5)	S (<=0.5)
B1905/18	I	(++)	(+)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (<=0.5)	S (<=0.5)
B1934/18	I	(++)	(+)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (=1)	S (<=0.5)
B042/19	I	(+++)	(+)	SIE (<=4)	SIE (=4)	SIE (=2)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (<=0.5)	S (<=0.5)
B048/19	I	(++)	(+)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (=1)	S (<=0.5)
B274/19	I	(++)	(++)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (=1)	S (<=0.5)
B084/20	II	(++)	(+)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (=1)	S (<=0.5)

Table 2. Cont.

Strain	Phenotypic Profile	Biofilm Formation		TZP ^b	CAZ ^c	CPM ^d	IPM ^e	Antibiogram (MIC ^a)		CIP ^h	TIG ⁱ	CST ^j
		22.5 °C	37.0 °C					MEM ^f	AMK ^g			
B167/20	I	(+)	(+)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (=1)	S (<=0.5)
B262/20	XI	(+++)	(+++)	SIE (=8)	SIE (=2)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (=1)	S (<=0.5)
B576/20	XVIII	(+++)	(++)	SIE (=8)	SIE (=4)	SIE (=1)	SIE (=1)	S (<=0.25)	S (<=2)	SIE (<=0.25)	R (>=8)	S (<=0.5)

^a—minimum inhibitory concentration; ^b—piperacillin-tazobactam; ^c—ceftazidim; ^d—cefepim; ^e—imipenem; ^f—meropenem; ^g—amikacin; ^h—ciprofloxacin; ⁱ—tigeciclin; ^j—colistin; ^k—moderate adherent; ^l—weak adherent; ^m—susceptible, increased exposure; ⁿ—susceptible; ^o—resistant; ^p—strong adherent.

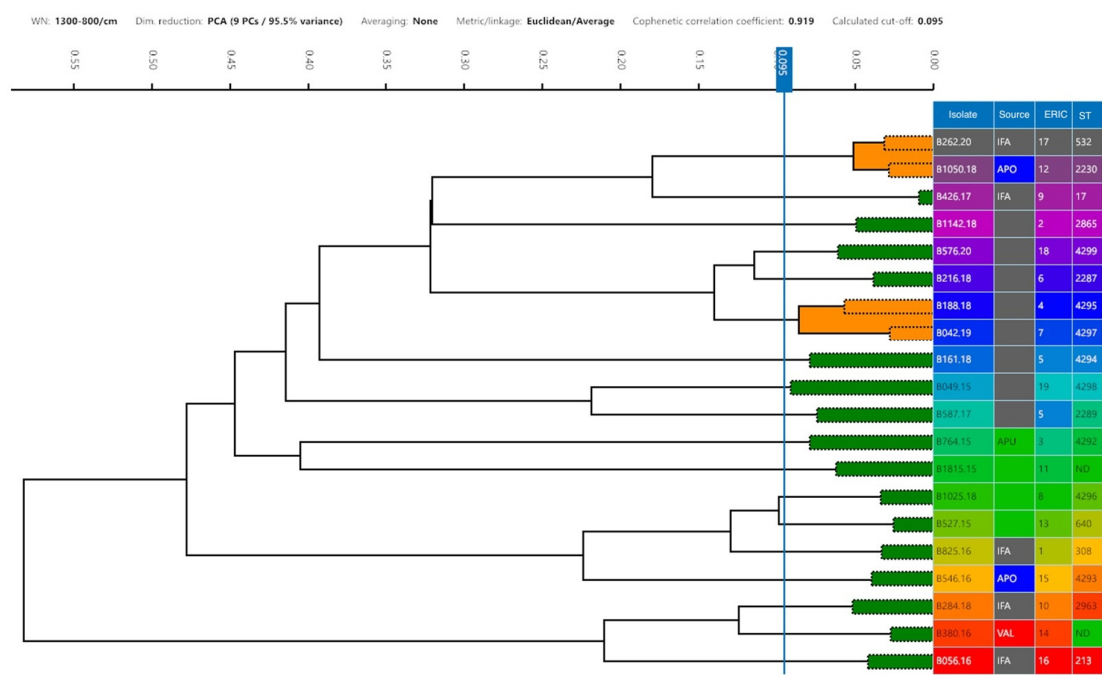


Figure 1. Dendrograms obtained by clustering FT-IR spectra for *P. aeruginosa* strains ($n = 20$). The blue vertical dashed lines represent the cut-off value (0.095). Green spectra indicate that the strain was a singleton, and orange spectra indicate a cluster formation. Different colors are used to differentiate the information regarding: isolate; source, enterobacterial repetitive intergenic consensus profile (ERIC), and sequence typing. (ST). Wavenumber (WN)—a quantity directly related to the frequency of electromagnetic radiation and defined as the inverse of the wavelength; principal component analysis (PCA).

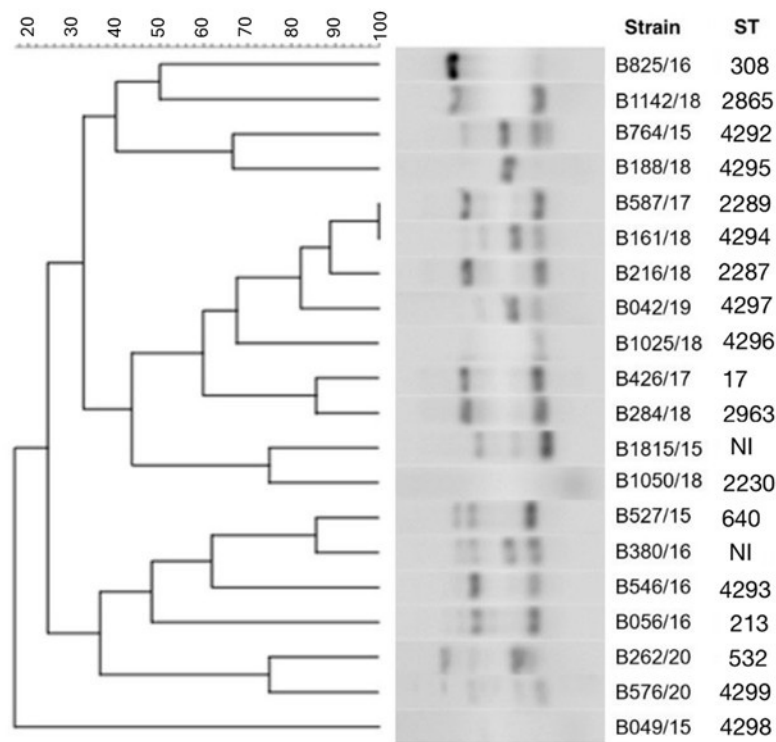


Figure 2. Dendrogram of the band profile representing the patterns obtained from *P. aeruginosa* strains ($n = 20$) by ERIC-PCR. Dendrogram obtained based on UPGMA clustering and similarity analysis using Dice coefficient calculation, constructed with BioNumerics 8.1 software (Applied Maths, bioMérieux). NI: Not identified; ST: Sequence type.

MLST analysis revealed that the isolates were in various sequence types (STs). These included 10 previously described STs: ST17, ST213, ST308, ST532, ST640, ST2230, ST2287, ST2289, ST2865, and ST2963. In addition, eight new STs were described in this study (ST4292–ST4299). The STs of strains B1815/15 and B380/16 could not be determined. The calculated SIs for ERIC-PCR and MLST were both 0.995 (0.981–1.000). The genetic relationships of the STs identified in this study that formed clonal complexes (CCs) with STs previously deposited in the PubMLST database are illustrated in Figure 3.

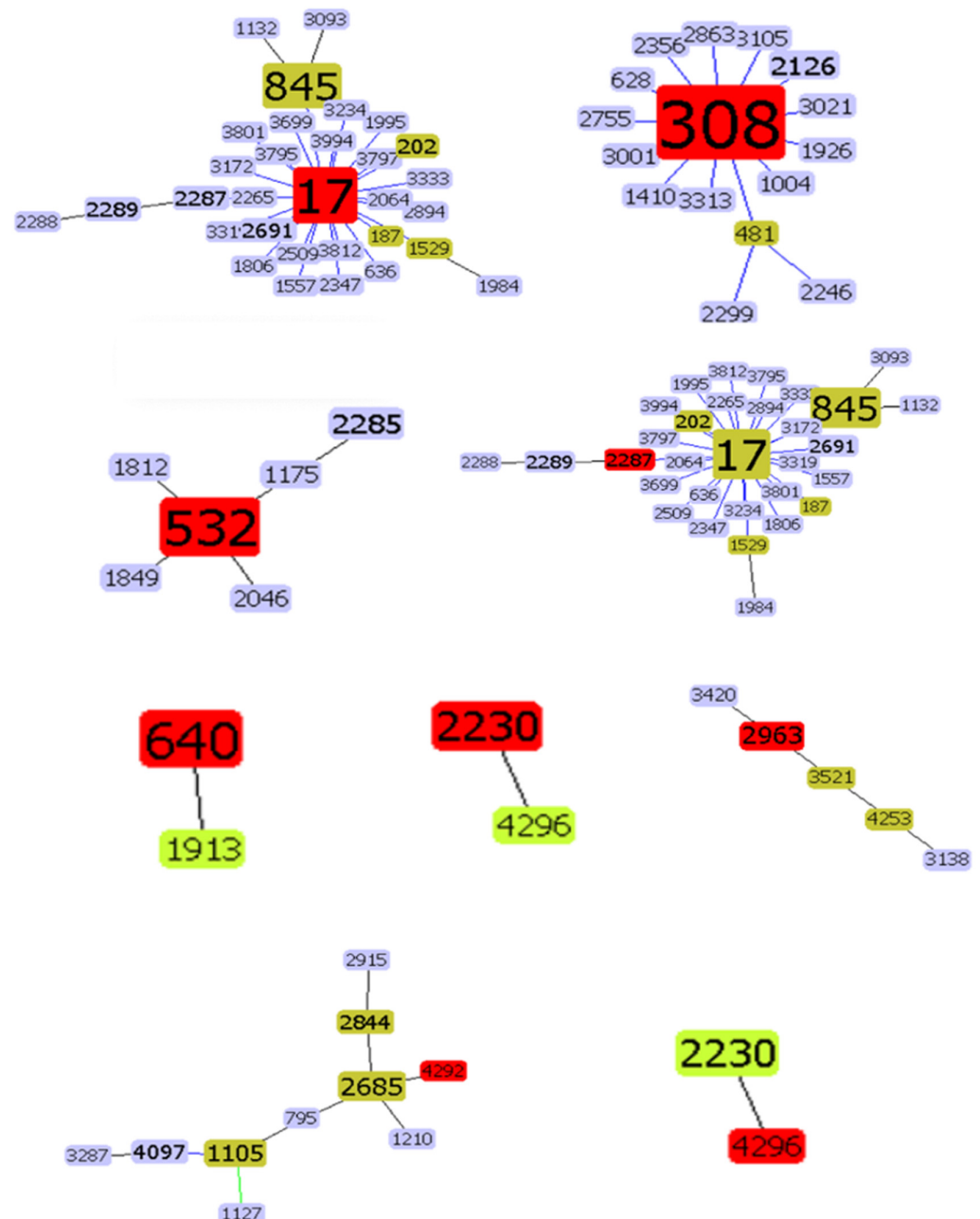


Figure 3. Minimum spanning tree of the clonal complexes of *P. aeruginosa* STs identified in this study (ST 308, 532, 640, 2230, 17, 2287, 2289, 2963, 4292, and 4296) indicated in red, along with the STs deposited in the PubMLST database, where other colors represent different sequence types and clonal complexes from the database. The figure was constructed using the PHYLOViz 2.0 software with the goeBURST algorithm, selecting the single locus variant (SLV) and double locus variant (DLV). The size of each number is proportional to the number of isolates belonging to that respective ST in the studied dataset. The lines connecting the STs indicate the similarity between them, based on the number of differing sequence loci distance.

3.2. Antimicrobial Susceptibility Evaluation

All *Pseudomonas* spp. ($n = 36$) strains showed sensitivity to amikacin ($\text{MIC} \leq 2$) and colistin ($\text{MIC} \leq 0.5$), and SIE to piperacillin/tazobactam (MIC ranging between 4 and 8) and ciprofloxacin ($\text{MIC} \leq 0.25$). All strains were resistant to tigecycline (MIC ranging from 0.5 to 8). Two (5.5%) strains exhibited resistance to ceftazidime ($\text{MIC} = 16$), while the remaining 34 (94.4%) showed SIE (MIC ranging between 1 and 8). Two (5.56%) strains showed resistance to cefepime ($\text{MIC} \leq 1$), with the rest demonstrating SIE (MIC ranging between 1 and 4). One (2.7%) strain was resistant to imipenem ($\text{MIC} \geq 16$) and showed SIE to meropenem ($\text{MIC} = 4$). The remaining strains ($n = 35$; 97.2%) displayed “Increased exposure sensitivity” to imipenem (MIC ranging from 0.002 to ≤ 2). All strains were classified as MDR due to resistance and/or SIE to more than five distinct classes of antimicrobials (aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillins + β -lactamase inhibitors).

3.3. Biofilm Formation and Antibiofilm Testing

In the statistical analyses, no significant difference ($p = 0.65$) was observed in biofilm formation between the temperatures of 22.5 ± 2.5 °C and 37.0 ± 2 °C. At 22.5 ± 2.5 °C, among the *P. aeruginosa* strains ($n = 20$), ten (50.0%) produced biofilm classified as strong adherent, six (30.0%) as moderate adherent, and four (20.0%) as weak adherents. Among the other non-*aeruginosa Pseudomonas* species ($n = 16$), twelve (75.0%) formed moderate adherent biofilm and four (25.0%) formed weak adherent biofilm. At 37 ± 2 °C, among the *P. aeruginosa* strains, five (25.0%) formed biofilm classified as strong adherent, seven (35.0%) as moderate adherent, and eight (40.0%) as weak adherents. Among the non-*aeruginosa Pseudomonas* species, one (6.2%) strain formed moderate adherent biofilm and the remaining 15 (93.8%) formed weak adherent biofilm (Table 2).

Of the 36 *Pseudomonas* spp. strains which were evaluated, 28 (77.8%) were classified as moderate or strong adherent and selected for the evaluation of biofilm tolerance to disinfectants. Exposure to 70% alcohol reduced the biofilm formed by nine (32.1%) strains (Figure 4) ($p = 0.035$). Exposure to quaternary ammonium at a concentration of 0.05% reduced the biofilm of 24 (85.7%) strains ($p = 1.3 \times 10^{-5}$). Of the nine strains that still exhibited moderate or strong adherence, a concentration of 0.08% quaternary ammonium reduced the biofilm of three (33.3%). Exposure to sodium hypochlorite at a concentration of 0.01% reduced or eliminated the biofilm of 24 (85.7%) strains ($p = 2.8 \times 10^{-7}$). The remaining strains ($n = 11$) that still showed moderate or strong adherent biofilm, but when exposed to hypochlorite at concentrations of 0.05, 1, 2, and 2.5%, their biofilm was reduced or eliminated, as shown in Figure 4.

3.4. Biofilm Morphological Analysis by Scanning Electron Microscopy (SEM)

P. aeruginosa biofilms were analyzed using scanning electron microscopy (SEM) to evaluate the cellular morphology and aggregation on stainless steel surfaces (Figures 5–8). All strains showed adherent cells on the surface, but five strains (B049/15, B284/18, B1050/18, B042/19, and B262/20) showed higher cell abundance and aggregation.

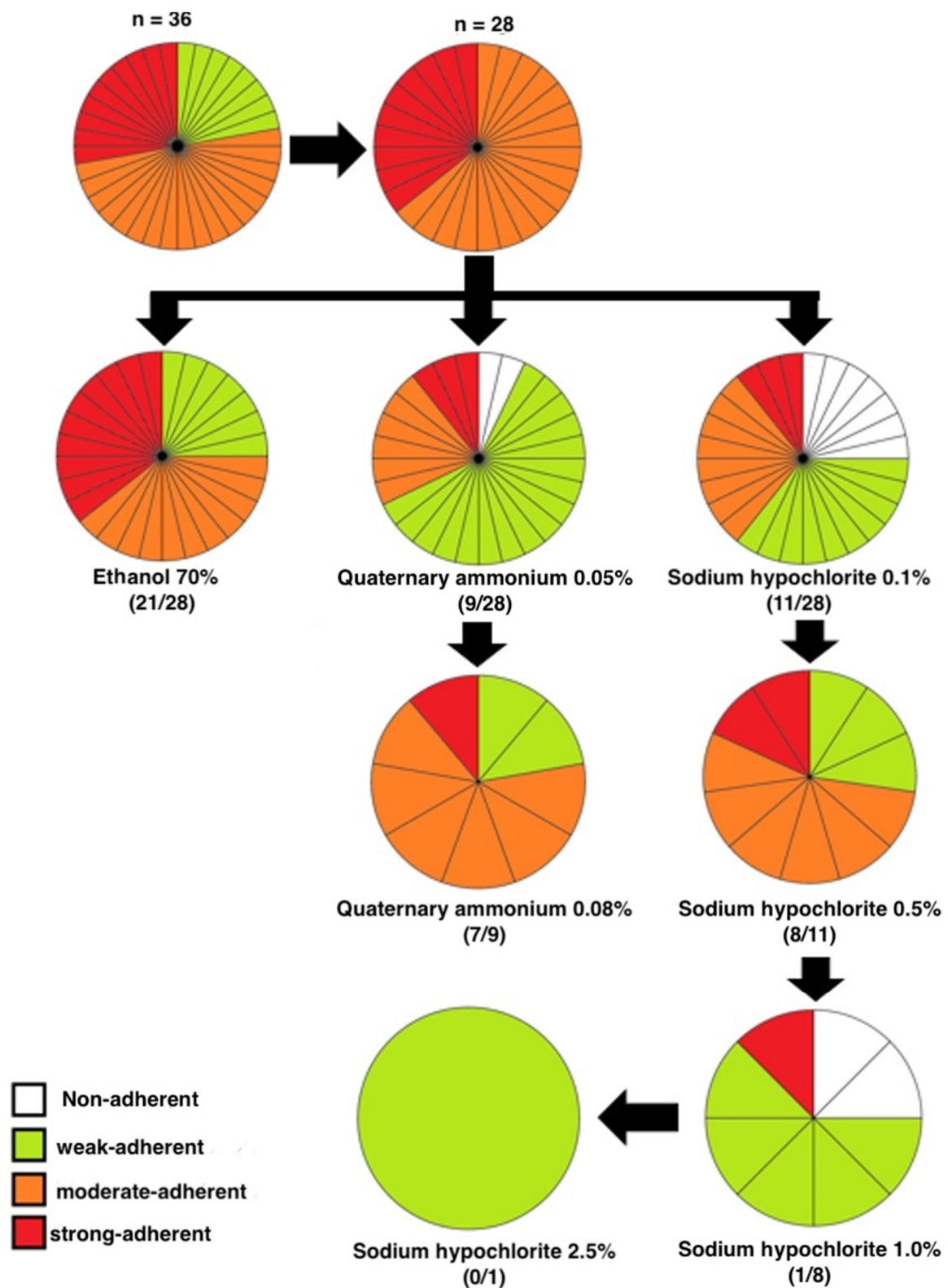


Figure 4. Evaluation of *Pseudomonas* spp. (n = 36) biofilm formation after exposure to disinfectants. The arrows indicate the reduction in biofilm for each isolate following exposure to disinfectants.

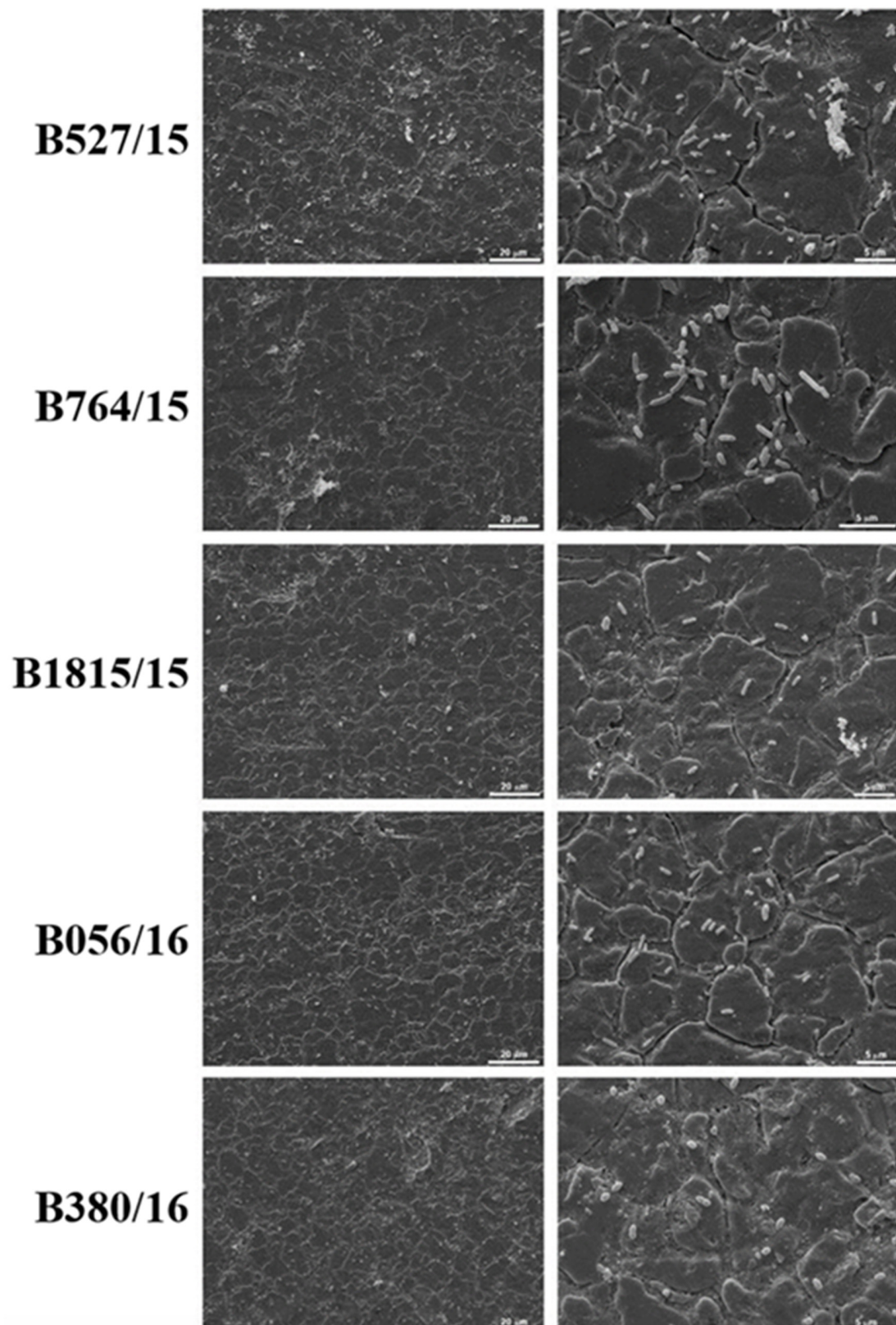


Figure 5. Morphological analysis of biofilm formation by different *P. aeruginosa* strains. Scanning electron microscopy reveals structural differences in biofilm architecture. The strains shown are B527/15, B764/15, B1815/15, B056/16, and B380/16. High magnification is displayed on the right (scale bar: 5 µm), and low magnification on the left (scale bar: 20 µm).

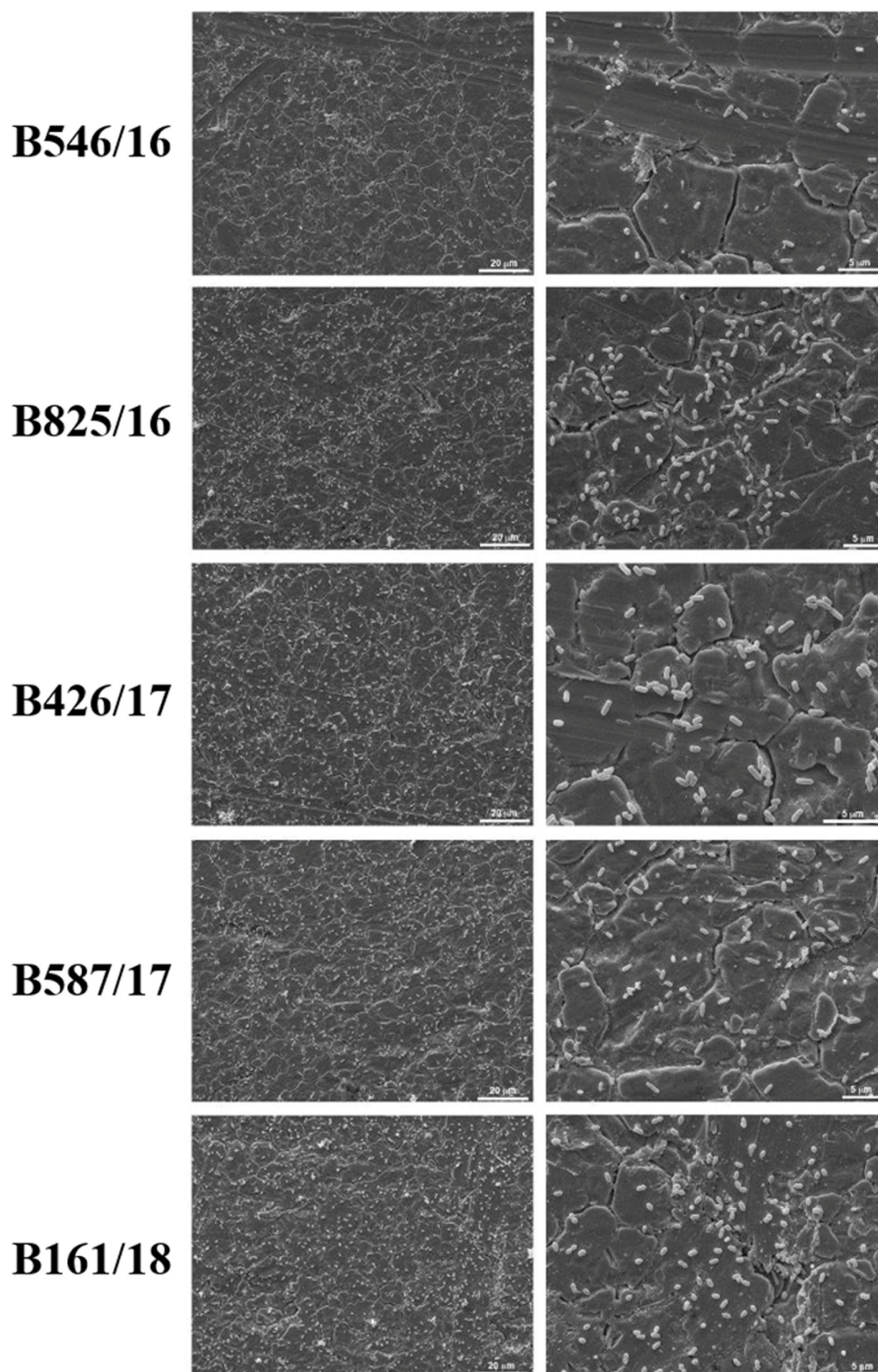


Figure 6. Morphological analysis of biofilm formation by different *P. aeruginosa* strains. Scanning electron microscopy highlights variations in biofilm structure. The strains shown are B546/16, B825/16, B426/17, B587/17, and B161/18. High magnification is displayed on the right (scale bar: 5 μm), and low magnification on the left (scale bar: 20 μm).

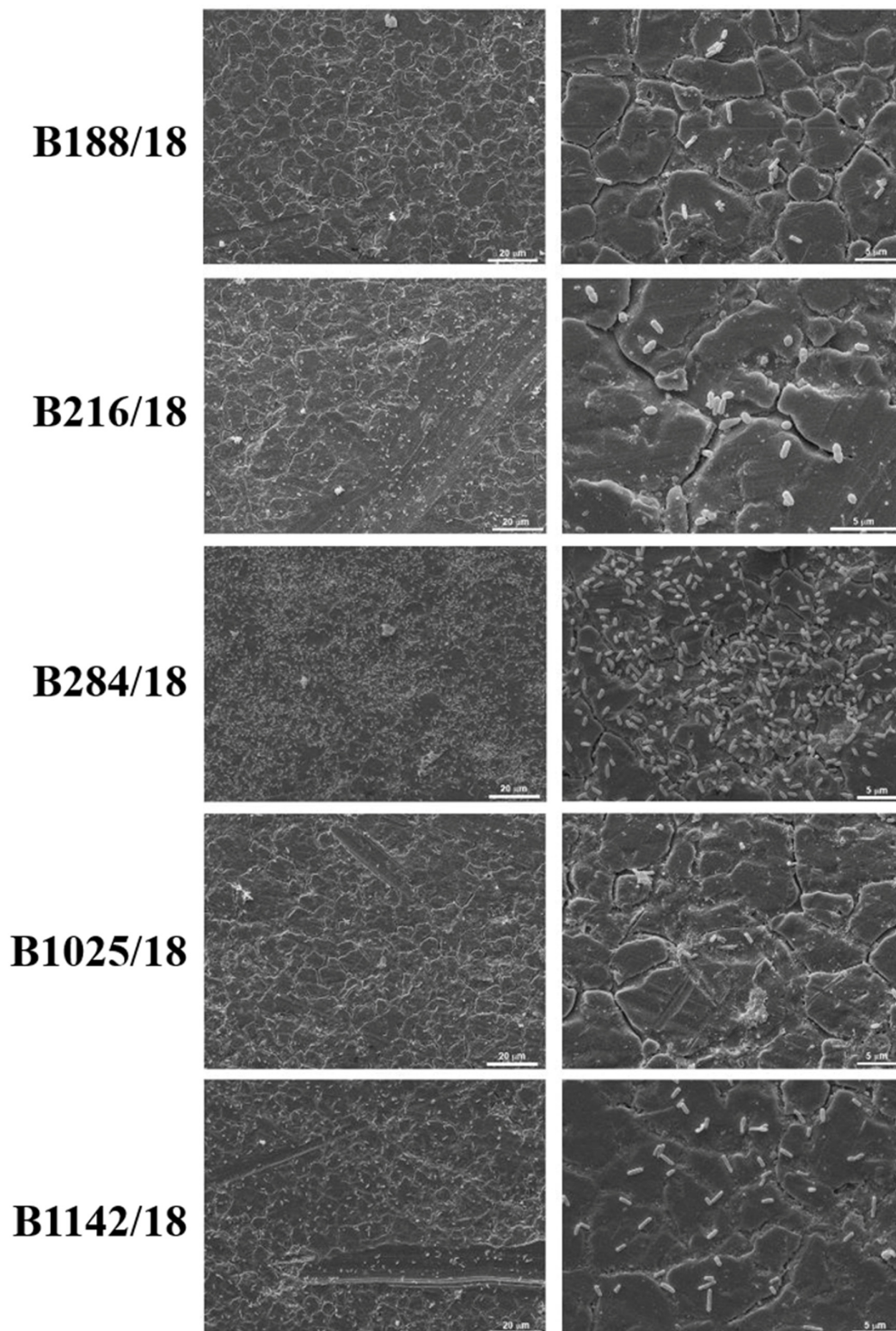


Figure 7. Morphological analysis of biofilm formation by different *P. aeruginosa* strains. Scanning electron microscopy provides insight into structural complexity within the biofilms. The strains shown are B188/18, B216/18, B284/18, B1025/18, and B1142/18. High magnification is displayed on the right (scale bar: 5 μm), and low magnification on the left (scale bar: 20 μm).

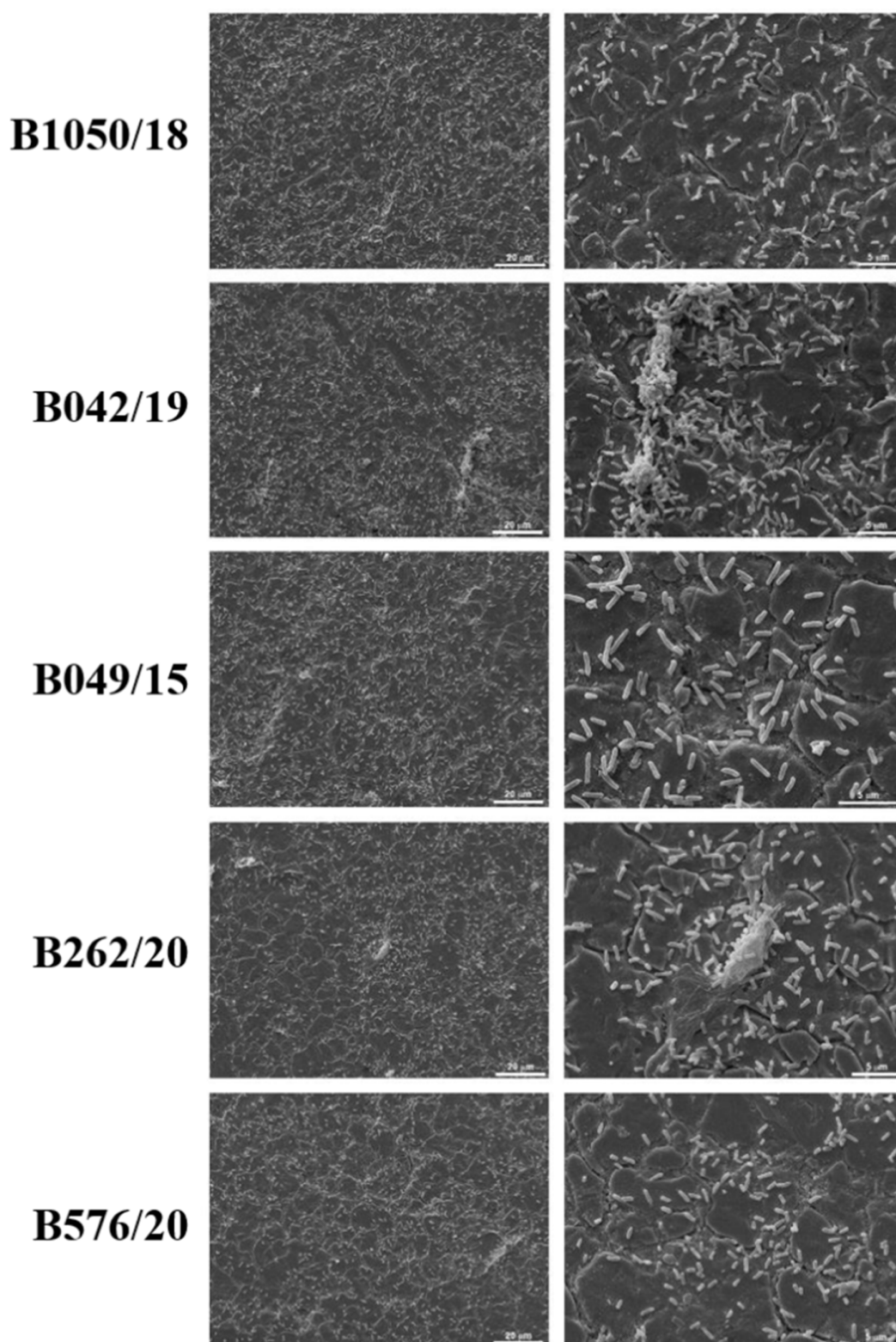


Figure 8. Morphological analysis of biofilm formation by different *P. aeruginosa* strains. Scanning electron microscopy captures distinct morphological traits across the biofilms. The strains shown are B1050/18, B042/19, B049/15, B262/20, and B576/20. High magnification is displayed on the right (scale bar: 5 µm), and low magnification on the left (scale bar: 20 µm).

4. Discussion

Correct bacterial identification plays a crucial role in the microbiological safety of pharmaceutical products for human use [33]. Contamination of these products by pathogenic

strains, such as *P. aeruginosa*, especially those resistant to antimicrobials and capable of forming biofilms, poses a substantial threat to the safety of immunobiologicals. In contrast, the 16 strains misidentified as *P. aeruginosa* could result in lot rejection, financial loss, and unnecessary investigations [34].

Strains of *P. aeruginosa* have been isolated in Brazil and around the world from various sources, such as clinical [12,35–37], environmental [13,16,38], and industrial [39]. In the present study, from the initial 40 strains, the *P. aeruginosa* strains (n = 20) were primarily isolated from sterility tests (12/20) and water samples (7/20) (Table 1).

The process of 16S rRNA gene sequencing is a traditional and widely used method for identifying *Pseudomonas* spp. strains, defining the prokaryotic taxonomy of the species [40]. In this study, four strains were identified as Gram-positive organisms: *Staphylococcus* and *Enterococcus* (Table 1). The Gram-staining characteristic (Gram-positive instead of Gram-negative) of the isolated strain was responsible for the misidentification by VITEK® 2, since the choice of the identification card will depend on the characteristics of the microorganism visualized in the Gram-staining [41]. These four strains were removed from further analysis.

Polyphasic analysis using VITEK® 2 confirmed that 20/36 strains were *P. aeruginosa*. VITEK® 2 showed 100% of sensitivity, 50.0% of specificity, and 69.2% of accuracy. Proteomic identification using VITEK® MS showed 91.7% of sensitivity, 100% of specificity, and 95.6% of accuracy. MALDI Biotyper® showed 91.2% of sensitivity, 86.7% of specificity, and 89.8% of accuracy. The B1142/18 strain was incorrectly identified as *P. koreensis* by MALDI Biotyper®, but the score obtained was 1.65, representing a low confidence in species identification. Strains B926/18 and B1151/18 were identified as *P. aeruginosa* by MALDI Biotyper® and VITEK® 2, but 16S rRNA gene sequencing identified them as different possible non-*P. aeruginosa* species. The B1815/15 strain was incorrectly identified as *P. koreensis* by MALDI Biotyper® (score 2.04) and not identified by VITEK® MS (Table 1).

Caldeira et al. [33] compared the VITEK® MS and MALDI Biotyper® systems for identifying 46 strains isolated from an immunobiological production facility. The strains were later subjected to 16S rRNA gene sequencing for confirmation and comparison of the sensitivity and specificity of both methods. The study concluded that VITEK MS® was unable to identify most strains (91.3%), while MALDI Biotyper® identified 34.8% of the strains. These results can be attributed to the database of each system, since the MALDI Biotyper® library possesses more aerobic endospore-forming bacteria species than the VITEK® MS. In the present study, the performance of both MALDI-TOF MS systems showed similar sensitivity, but VITEK MS® presented higher specificity, since MALDI Biotyper® incorrectly identified three strains as *P. aeruginosa* (Table 1).

The possible non-*P. aeruginosa* species identified by 16S rRNA sequencing included 46 different species (Table 1). Among the species that presented the higher % of similarity, *P. koreensis* has been isolated from soil, animals, and food [42,43]. *P. atacamensis* and *P. maraviensis* have been isolated from soil and plants [44,45], and *P. mucoides* from animals and soil [46,47]. In this study, 16 strains (40.0%) were identified by 16S rRNA gene sequencing as one of these cited species. To date, there are no reports in the literature of these *Pseudomonas* species in the pharmaceutical industry.

From the 20 *P. aeruginosa* strains submitted to typing, only 18 could be assigned to existing STs. ST 640 has been previously isolated from blood and water samples in the Czech Republic and France and is an SLV of ST1913, also isolated from a clinical sample (sputum) in Spain in 2013. ST17 has 90 strains deposited and has been isolated from clinical specimens (blood, bronchoalveolar lavage, urinary tract infection, sputum, and soft tissue infection) and water, between 1986 and 2020 in many countries, forming a CC with 6437 isolates, divided into 2398 distinct STs, forming SLVs with 750 strains. STs 17, 308, and 532 belong to this same CC, with isolates of clinical and environmental origin

(water). ST4296, identified in this study, is an SLV of ST2230, identified from clinical samples (otitis) in the USA (Figure 3). These results may indicate that the supply water, which is primarily used within the pharmaceutical industry, could be a source of dissemination for these clones, which are present even in pharmaceutical production environments. No *P. aeruginosa* strains isolated from pharmaceutical industries had been deposited in the PubMLST database until this study (accessed on 5 January 2026).

Eight new STs (ST4292–4299) were described. This finding confirms that the pharmaceutical industry environment is an important niche that still needs to be explored to fully understand the epidemiology of this pathogenic species. This result was similar to a previously published work that characterized *Acinetobacter* spp. strains from a pharmaceutical industry facility and identified nine new STs [10].

The ST of two strains (B1815/15 and B380/16) could not be determined. This may occur because the primers in the database might not be sufficiently specific for environmental strains, possibly due to variations in the regions where they bind.

After FT-IR analysis, strains B262/20 and B1050/18 clustered together but had distinct STs (ST532 and ST2230, respectively). Both strains were also isolated from different samples (sterility test and APO) and in different years (2020 and 2018) (Table 1). Strains B188/18 and B042/19 also clustered together and had distinct STs (ST4295 and ST4297), both isolated from sterility tests but in different years (2018 and 2019) (Table 1). These new STs were identified in this study and did not form CCs with other STs identified. Strains B587/17 and B161/18 showed 100.0% similarity in ERIC-PCR (Figure 2) but differed in their FT-IR profiles and had distinct STs (ST2289 and ST4294) (Figure 1). FT-IR has been used for the identification and differentiation of *P. aeruginosa* strains isolated from clinical samples [1,48] but had not been used for typing strains isolated from pharmaceutical industries. The presence of *P. aeruginosa* is considered critical in bioburden testing, water analysis, and raw materials within the industry [3,5]; thus, accurate identification of this microorganism is crucial for ensuring the quality of immunobiological products.

Pseudomonas spp. strains showed resistance to different antimicrobials (Table 2). Following the classification proposed by Magiorakos et al. [11], all strains were classified as MDR because they showed resistance and/or “susceptible, increased exposure” to more than five distinct classes of antimicrobials (aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillins + β -lactamase inhibitors). Therefore, it is necessary to monitor and implement control measures to eliminate these strains from the pharmaceutical industry environment. The spread of resistant *P. aeruginosa* strains is a widely discussed problem in the literature, and its research is even more important with the growing One Health approach [49]. The increased use of antibiotics during the COVID-19 pandemic as prophylaxis in severely hospitalized patients may have further contributed to the development and spread of antibiotic-resistant microorganisms (cephalosporins and carbapenems), including *P. aeruginosa* [50,51]. This large-scale empirical antibiotic prescription during the pandemic exacerbated public health issues [50,52–54]. To prevent the spread of MDR *P. aeruginosa* strains, infection control measures and antibiotic administration can be improved through a deep understanding of local resistance patterns and the prevalence of carbapenemase genes [51]. Given the need for broad-spectrum antibiotics in cases of *P. aeruginosa* infections, the environmental spread of MDR *P. aeruginosa* strains found in this study is a public health issue and the need for monitoring and controlling these strains within the pharmaceutical industry must be discussed.

Despite advances in the treatment of biofilm-related infections by pathogenic strains such as *P. aeruginosa*, morbidity and mortality rates remain high [55,56] due to the antimicrobial resistance present in these strains. Microbial cells within a biofilm are less susceptible to antimicrobials and more resistant to host immune system attacks [57]. Inhi-

bition of biofilm formation is a viable approach to improve antimicrobial efficacy [57]. In this study, 28 (77.8%) *Pseudomonas* spp. strains formed biofilms on polystyrene surfaces and were classified as moderate adherent or strong adherent (Table 2). All *P. aeruginosa* strains evaluated ($n = 20$) could form biofilm on stainless steel surfaces (Figures 5–8). In biofilm tolerance testing against disinfectants, among the disinfectants evaluated, exposure to sodium hypochlorite at a concentration of 0.01% reduced or eliminated biofilm from 24 (85.7%) strains. The remaining strains ($n = 11$) that still presented moderate or strong adherent biofilm, when exposed to sodium hypochlorite at concentrations of $\geq 0.05\%$, had their biofilm reduced or eliminated (Figure 4). Vasconcellos et al. [10] evaluated three disinfectant agents (70% alcohol, sodium hypochlorite, and quaternary ammonium) against biofilm from *Acinetobacter* spp. strains isolated from a pharmaceutical industry and observed that only 0.1% hypochlorite/15 min was effective. Miranda et al. [8] tested the efficacy of disinfectants against biofilms formed by *S. maltophilia* and concluded that sodium hypochlorite at concentrations $\geq 1.0\%/15$ min appears to be the most effective disinfectant for eliminating these strains. Since sodium hypochlorite cannot be used on certain surfaces due to its corrosive action, further studies are needed to find alternative disinfectants. Disinfection processes within pharmaceutical industries must be constantly evaluated [58], as the presence of and failure to eradicate biofilm-forming strains can harm the production chain and contaminate the final product [7].

5. Conclusions

In conclusion, after the polyphasic characterization of the 40 strains previously identified as *P. aeruginosa*, VITEK[®]MS was shown to be the best methodology for the accurate identification of *P. aeruginosa* strains. All *P. aeruginosa* strains were classified as MDR. Sodium hypochlorite at concentrations of $\geq 0.05\%$ was the best disinfectant for biofilm reduction or elimination.

Whole-genome sequencing of these lineages in future studies has the potential to elucidate the molecular bases of the resistance phenotypes identified and the genes related to biofilm formation.

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Data Availability Statement: The sequencing data for the 16S rRNA gene, which support the findings of this study, have been deposited in the National Center for Biotechnology Information (NCBI) repository. Accession numbers are listed in Table 1. All other data are included within this article.

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Conflicts of Interest: Stephen James Forsythe is employed by Foodmicrobe.com Ltd., the remaining authors declare that they have no conflicts of interest.

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