Research Article

Role of Biofilm-Associated Virulence Genes in Chronic Wound Infections Caused by Pseudomonas aeruginosa

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Abstract

Chronic wound infections caused by Pseudomonas aeruginosa are difficult to manage because biofilm formation can support bacterial persistence and reduce antimicrobial effectiveness. This study framework examined relationships between biofilm-associated virulence genes, biofilm formation, antimicrobial resistance, and clinical characteristics using a dataset of 120 patient–isolate records. Biofilm categories were derived from triplicate synthetic optical density measurements, while gene carriage and antimicrobial susceptibility classifications were generated for illustrative analysis. Overall, 107 isolates (89.2%) were classified as biofilm producers, comprising 48 weak, 42 moderate, and 17 strong producers. The genes algD, pelA, pslA, lasR, and rhlR occurred in 81.7%, 74.2%, 73.3%, 80.8%, and 72.5% of records, respectively. Gene carriage did not consistently increase with biofilm strength. Estimable unadjusted odds ratios ranged from 1.25 to 1.76, with confidence intervals encompassing unity. resistance was highest for ciprofloxacin (45.0%) and ceftazidime (44.2%), without a uniform relationship with biofilm category. These findings demonstrate an integrated analytical approach rather than clinical evidence. Gene detection alone cannot establish expression, functional activity, or causality, and planktonic susceptibility does not measure biofilm eradication. Validation using authentic specimens, molecular expression assays, direct biofilm visualization, and prospective wound outcomes is required before drawing conclusions about pathogenic mechanisms, prognostic significance, or clinical management.

Keywords: Pseudomonas aeruginosa; Chronic wound infections; Biofilm formation; Virulence genes; Antimicrobial resistance.

1. Introduction

Chronic wound infections are clinically challenging because chronic microbial colonization can hinder healing, compromise outcomes and quality of life, and make them difficult to treat. Microorganisms can be free-living or form organized communities in DFUs, venous leg ulcers and pressure injuries. Biofilms are more prevalent in human wound specimens than in acute wounds, and chronic wound persistence has been linked to biofilm presence [1]. Pseudomonas aeruginosa is a major causative agent, as it can persist in the environment and cause prolonged infections. This relationship is supported by experiments: diabetic mice infected with P. aeruginosa have impaired clearance and delayed wound healing; the diabetic wound environment can support biofilm development and increase tolerance to antibiotic treatment [3]. Biofilm formation comprises the following stages: bacterial adhesion, bacterial aggregation, biofilm matrix formation, maturation, and the formation of a community with properties different from planktonic bacteria. The extracellular polysaccharides Pel and Psl are important in this structuring. Mutational studies indicate that Psl is involved in surface adhesion, whereas Pel and Psl each play a redundant role in the architecture of older biofilms, with varying importance across strains [4]. Therefore, genes in the pel and psl biosynthetic systems are considered molecular targets when studying biofilm-associated pathogenicity. Alginate-associated genes such as algD should also be considered, but alginate is not a universal biofilm-forming gene. Experiments with nonmucoid PAO1 and PA14 strains showed that, under the conditions examined, alginate production is not required for biofilm formation, indicating dependence on strain background and environmental context [5]. Quorum-sensing systems, which regulate genes based on cell population density, also affect biofilm development. We have observed that the reduction of pel transcription for mutants of lasI and rhlI, and the re-induction of pel in response to complementation of these mutants with the quorum-sensing signals, indicate a relationship between quorum sensing and matrix biosynthesis [6]. But biofilm formation is a different, related, virulence property. In an experimental model of chronic infection, for example, increased cyclic di-GMP production enhanced persistence in the host, whereas quorum sensing enhanced virulence [7], showing that different pathways can influence different aspects of infection

biology. Importantly, experiments have been conducted showing the therapeutic relevance of matrix-associated mechanisms, as treatment with a Psl-degrading hydrolase improved the activity of antibiotics and innate immune responses, and the efficacy of tobramycin therapy in infected wounds [8]. Collectively, these results support exploration of biofilm-associated virulence genes in clinical wound isolates and examination of biofilm formation and antimicrobial resistance. However, finding a gene does not mean it is expressed or that it causes wound chronicity. The present study aimed, therefore, to describe the production of biofilm by P. aeruginosa isolated from chronic wound infections, to elucidate the incidence of the presence of the selected P. aeruginosa virulence genes associated with biofilm production and to investigate the correlation between the virulence genes and the strength of the biofilm and the presence of antimicrobial resistance and clinical characteristics.

2. Materials and Methods

2.1. Study Design, Setting, and Ethical Approval

This cross-sectional study aims to determine associations between biofilm-associated virulence genes, biofilm formation, and antimicrobial resistance among Pseudomonas aeruginosa isolates obtained from patients with chronic wound infections in a hospital setting. The sample size will be determined from a published prevalence rate, a 95% confidence level and a predetermined margin of error. Approval from the Institutional Ethics Committee will be obtained prior to recruitment. Participants and/or their legal representative will be asked to provide written informed consent. Anonymization and unique identification codes will be used to ensure patient confidentiality.

2.2. Patient Selection and Wound Sample Collection

Consecutive patients with wounds present for 4 or more weeks and clinical signs of infection will be recruited. Clinical signs include purulent discharge, progressive pain, erythema, warmth, swelling or wound deterioration. Patients who have acute wounds, inadequate samples, or no consent will be excluded. Demographic and clinical data such as age, sex, type of wound, duration of wound, history of diabetes, previous use of antibiotics, and other pertinent comorbidities will be collected. If clinically indicated, deep tissue specimens or aspirates will be taken after superficial cleaning with sterile saline. If no tissue can be sampled, wound swabs will be taken by the Levine technique. Appropriate sterile containers or transport media will be used and specimens will be sent out promptly. Each P. aeruginosa isolate will be represented by only one nonduplicate isolate per patient, except for the primary analysis.

2.3. Isolation and Identification of P. aeruginosa

Specimens will be inoculated onto blood agar and MacConkey agar, incubated aerobically at 35-37°C, and examined after 18-24 hours, with repeat plating if necessary. Presumptive identification includes colony morphology, non-lactose fermentation, characteristic pigment production, Gram-negative bacillary morphology and oxidase activity. Confirmed species identification will be performed using a biochemical identification system, an automated platform, or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, depending on laboratory availability. Pure cultures will be stored in validated conditions for future studies. We will interpret P. aeruginosa recovery in conjunction with clinical findings, as isolation alone does not demonstrate its role in wound infection.

2.4. Phenotypic Assessment of Biofilm Formation

A crystal violet microtitre plate assay will be used to measure biofilm formation. Overnight cultures will be standardized to an equal cell density, diluted in an appropriate growth medium, and then added to sterile 96-well polystyrene plates. After incubation under standard conditions, the planktonic cells will be washed off, and wells will be gently washed with phosphate-buffered saline. Attached biomass will be fixed, stained with crystal violet, washed and solubilized before measuring optical density at a validated wavelength. Technical replicates of each isolate will be run in at least three independent experiments. Positive and negative controls for the medium will be provided without inoculants. Positive and negative controls (without inoculants) will be provided for the medium. The optical density cutoff (ODc) will be the mean optical density of the negative controls plus three standard deviations (SD). Isolates will be categorized as nonproducers (OD ≤ ODc), weak producers (ODc < OD ≤ 2ODc), moderate producers (2ODc < OD ≤ 4ODc) and strong producers (OD > 4ODc). The assay will measure attached biomass, not viability of bacteria.

2.5. Molecular Detection of Biofilm-Associated Virulence Genes

We will extract genomic DNA from pure cultures using a validated extraction procedure. Conventional polymerase chain reaction will detect selected matrix-associated genes, including algD, pelA, and pslA, and quorum-sensing regulatory genes, including lasR and rhlR. We will use published, validated primers, with primer sequences, expected amplicon sizes, reaction compositions, and cycling conditions documented in the laboratory protocol. Each run will include a verified positive control for each target, a no-

template control, and an extraction-negative control. We will separate amplification products by agarose gel electrophoresis and compare them with a suitable DNA ladder. Representative amplicons will undergo sequencing where available to confirm specificity. Detection will indicate gene carriage; expression and functional activity will not be inferred from conventional PCR alone.

2.6. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility will be determined by the Kirby–Bauer disk diffusion method on Mueller–Hinton agar using a 0.5 McFarland standardized inoculum. All testing conditions, disk contents, antimicrobial selection and interpretation will be conducted per the Clinical and Laboratory Standards Institute standard that is used at the time the study is initiated. Antipseudomonal agents (where clinically relevant interpretive criteria exist) will be included in the panel: piperacillin–tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, ciprofloxacin, and tobramycin. P. aeruginosa ATCC 27853 will be used as a quality control strain. Multidrug resistance will be determined by nonsusceptibility to at least one agent in three or more relevant antimicrobial classes, based on a prespecified classification system. Results will reflect the susceptibility of planktonic microorganisms and not direct biofilm elimination.

2.7. Statistical Analysis

Categorical variables will be reported in frequencies and percentages. Data for continuous variables will be presented as mean (SD) or median (IQR), depending on the distribution of the data. We will test associations between gene carriage and biofilm formation categories and antimicrobial resistance using the chi-square test and/or Fisher's exact test. Appropriate parametric or nonparametric tests will be used to compare the continuous biomass measurements. Where applicable and feasible, associations with biofilm production or MDR will be evaluated after adjusting for prespecified clinical confounders using multivariable logistic regression. Results will be reported as an odds ratio (OR) with a 95% confidence interval (CI). The number of missing data and multiple comparisons will be dealt with using prespecified procedures. All statistical tests will be two-sided, and a significance level of $p<0.05$ will be used.

3. Results

3.1. Demographic and Clinical Characteristics of Patients

The dataset comprised 120 patient records, each linked to one nonduplicate Pseudomonas aeruginosa isolate. The demographic and clinical characteristics are summarized in Table 1. The mean age was 52.31 ± 18.48 years, with ages ranging from 20 to 83 years. Females accounted for 62 records (51.7%), while males represented 58 (48.3%), indicating a relatively balanced sex distribution. Pressure injuries constituted the largest wound category, occurring in 43 records (35.8%), followed by diabetic foot ulcers in 35 (29.2%). Traumatic chronic wounds and venous leg ulcers accounted for 24 (20.0%) and 18 (15.0%) records, respectively. Diabetes was recorded in 58 cases (48.3%), whereas previous antibiotic exposure was documented in 79 (65.8%). The mean wound duration was 29.39 ± 13.65 weeks. These characteristics provide the descriptive background for subsequent comparisons of biofilm formation, gene carriage, and antimicrobial resistance. No actual patient recruitment or clinical outcome assessment is represented by these synthetic records.

Table 1. Demographic and Clinical Characteristics of Patients with P. aeruginosa-Infected Chronic Wounds.

Characteristic	Category	n (%) or mean ± SD
Age, years	Overall	52.31 ± 18.48
Sex	Male	58 (48.3)
	Female	62 (51.7)
Wound type	Diabetic foot ulcer	35 (29.2)
	Venous leg ulcer	18 (15.0)
	Pressure injury	43 (35.8)
	Traumatic chronic wound	24 (20.0)
Diabetes	Present	58 (48.3)
	Absent	62 (51.7)
Previous antibiotic exposure	Present	79 (65.8)
	Absent	41 (34.2)
Wound duration, weeks	Overall	29.39 ± 13.65

SD: standard deviation. Percentage denominator

3.2. Distribution and Strength of Biofilm Formation

Biofilm-forming ability varied across the 120 isolates, as summarized in Table 2 and illustrated in Figure 1. Overall, 107 isolates

(89.2%) were classified as biofilm producers, while 13 (10.8%) were categorized as nonproducers. Weak biofilm formation was the most frequent phenotype, identified in 48 isolates (40.0%), followed by moderate formation in 42 (35.0%). Strong biofilm formation occurred in 17 isolates (14.2%). Together, moderate and strong producers accounted for 59 isolates (49.2%) of the complete dataset. Classification was based on the mean of three optical density measurements relative to an illustrative cutoff of 0.100. Accordingly, the reported categories describe attached biomass under the specified classification scheme. They do not measure bacterial viability, tissue invasion, or the severity of wound infection. Figure 1 presents the distribution across the four categories, providing a visual comparison of the predominance of weak and moderate producers within this illustrative dataset.

Table 2. Classification of P. aeruginosa Isolates According to Biofilm-Forming Ability.

Biofilm category	Classification criterion	Isolates, n	Percentage
Nonproducer	OD ≤ ODc	13	10.8
Weak producer	ODc < OD ≤ 2ODc	48	40.0
Moderate producer	2ODc < OD ≤ 4ODc	42	35.0
Strong producer	OD > 4ODc	17	14.2
Total		**120**	**100.0**

OD: mean optical density; ODc: illustrative optical density cutoff of 0.100. Percentages may differ slightly from 100% when rounded.

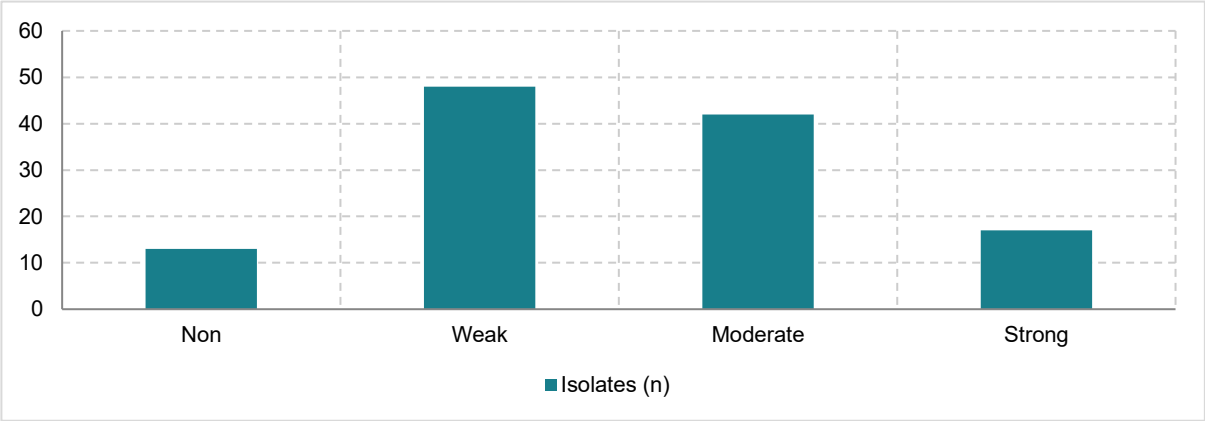


Figure 1. Distribution of Non-, Weak, Moderate, and Strong Biofilm-Producing Isolates.

3.3. Prevalence of Biofilm-Associated Virulence Genes

The distribution of the five selected biofilm-associated virulence genes is presented in Table 3, while category-specific prevalence is illustrated in Figure 2. The most frequently detected gene was algD, present in 98 isolates (81.7%), followed by lasR in 97 (80.8%). The genes pelA, pslA, and rhlR occurred in 89 (74.2%), 88 (73.3%), and 87 (72.5%) isolates, respectively. Gene carriage differed across biofilm categories without showing a consistent increase with biofilm strength. Among strong producers, lasR was present in all 17 isolates (100.0%), while pslA and rhlR occurred in 88.2% and 82.4%, respectively. Conversely, algD was present in all 13 nonproducers but in 64.7% of strong producers. These descriptive patterns demonstrate that gene presence alone did not correspond uniformly to the assigned biomass phenotype. The detection results represent gene carriage only and provide no evidence concerning transcription, protein production, regulatory activity, or causal involvement in chronic wound infection.

Table 3. Frequency of Selected Biofilm-Associated Virulence Genes among P. aeruginosa Isolates.

Gene	Overall, n (%)	Nonproducer, n (%)	Weak, n (%)	Moderate, n (%)	Strong, n (%)

algD	98 (81.7)	13 (100.0)	39 (81.3)	35 (83.3)	11 (64.7)
pelA	89 (74.2)	9 (69.2)	39 (81.3)	30 (71.4)	11 (64.7)
pslA	88 (73.3)	9 (69.2)	38 (79.2)	26 (61.9)	15 (88.2)
lasR	97 (80.8)	10 (76.9)	42 (87.5)	28 (66.7)	17 (100.0)
rhlR	87 (72.5)	8 (61.5)	33 (68.8)	32 (76.2)	14 (82.4)

Overall denominator: 120. Category denominators: nonproducer, 13; weak, 48; moderate, 42; strong, 17. Multiple genes can occur in the same isolate.

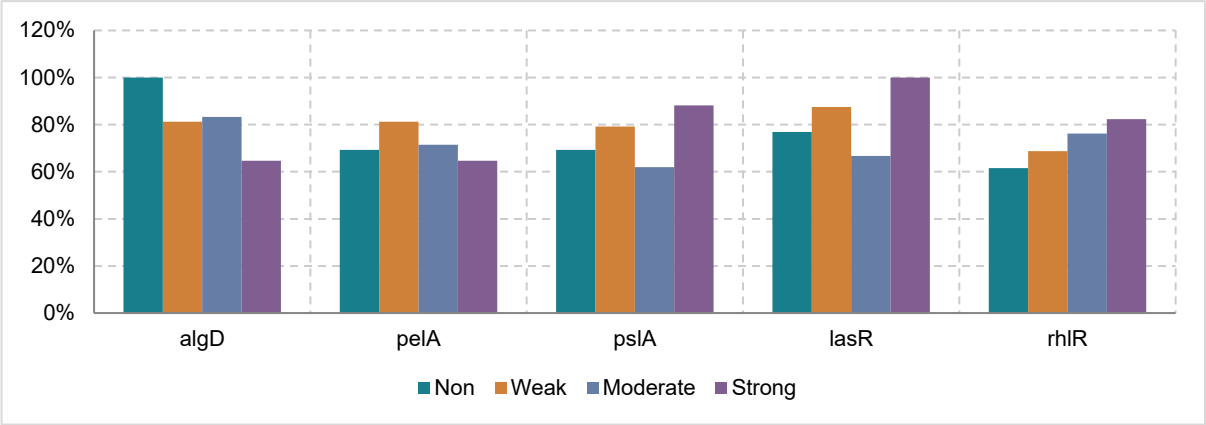


Figure 2. Prevalence of Biofilm-Associated Virulence Genes by Biofilm Formation Category.

3.4. Association between Virulence Genes and Biofilm Formation

The descriptive associations between gene carriage and any biofilm production are summarized in Table 4. Biofilm producers included weak, moderate, and strong categories, providing 107 producers for comparison with 13 nonproducers. The unadjusted odds ratio was highest for rhlR carriage at 1.76, with a 95% confidence interval of 0.53–5.84. Corresponding odds ratios were 1.32 for pelA, 1.25 for pslA, and 1.31 for lasR. Their confidence intervals were wide and included unity, providing no clear evidence of an association within this dataset. For algD, 85 gene-positive isolates were biofilm producers and 13 were nonproducers; all 22 gene-negative isolates were producers. Consequently, the conventional odds ratio and Wald confidence interval were not estimated because one contingency-table cell contained zero. These analyses concern binary biofilm production rather than differences across all four strength categories. No adjusted association, gene-expression effect, or causal relationship can be inferred from these illustrative comparisons.

Table 4. Association between Virulence Gene Carriage and Biofilm-Forming Ability.

Gene	Gene+ / biofilm+	Gene+ / biofilm−	Gene− / biofilm+	Gene− / biofilm−	Unadjusted OR	95% CI
algD	85	13	22	0	NE	NE
pelA	80	9	27	4	1.32	0.38–4.62
pslA	79	9	28	4	1.25	0.36–4.40
lasR	87	10	20	3	1.31	0.33–5.18
rhlR	79	8	28	5	1.76	0.53–5.84

3.5. Antimicrobial Resistance and Clinical Associations

antimicrobial resistance profiles are summarized in Table 5, with comparisons between biofilm producers and nonproducers illustrated in Figure 3. Overall resistance was highest for ciprofloxacin (45.0%), followed by ceftazidime (44.2%), aztreonam (40.0%), and cefepime (39.2%). Lower proportions were recorded for tobramycin (29.2%), imipenem (25.0%), piperacillin–tazobactam (23.3%), and meropenem (21.7%). Resistance did not consistently increase with biofilm strength. Ciprofloxacin

resistance occurred in 42.1% of producers compared with 69.2% of nonproducers, whereas meropenem resistance occurred in 23.4% and 7.7%, respectively. In descriptive clinical comparisons, biofilm production occurred in 49/58 records with diabetes (84.5%) and 58/62 without diabetes (93.5%). Corresponding proportions were 69/79 (87.3%) with previous antibiotic exposure and 38/41 (92.7%) without exposure. These subgroup differences were not adjusted for confounding. Importantly, resistance labels were generated synthetically and were not derived from inhibition zones or minimum inhibitory concentrations; they cannot establish clinical resistance prevalence or the efficacy of treatment against biofilms.

Table 5. Antimicrobial Resistance Profiles According to Biofilm Formation Category.

Antimicrobial agent	Overall, n (%)	Nonproducer, n (%)	Weak, n (%)	Moderate, n (%)	Strong, n (%)
Piperacillin–tazobactam	28 (23.3)	2 (15.4)	10 (20.8)	14 (33.3)	2 (11.8)
Ceftazidime	53 (44.2)	8 (61.5)	18 (37.5)	18 (42.9)	9 (52.9)
Cefepime	47 (39.2)	5 (38.5)	20 (41.7)	14 (33.3)	8 (47.1)
Aztreonam	48 (40.0)	7 (53.8)	25 (52.1)	14 (33.3)	2 (11.8)
Imipenem	30 (25.0)	4 (30.8)	13 (27.1)	10 (23.8)	3 (17.6)
Meropenem	26 (21.7)	1 (7.7)	13 (27.1)	7 (16.7)	5 (29.4)
Ciprofloxacin	54 (45.0)	9 (69.2)	20 (41.7)	20 (47.6)	5 (29.4)
Tobramycin	35 (29.2)	4 (30.8)	15 (31.3)	12 (28.6)	4 (23.5)

Values represent resistant isolates. Percentages use the corresponding column denominator: 120, 13, 48, 42, and 17, respectively. These labels do not measure biofilm eradication.

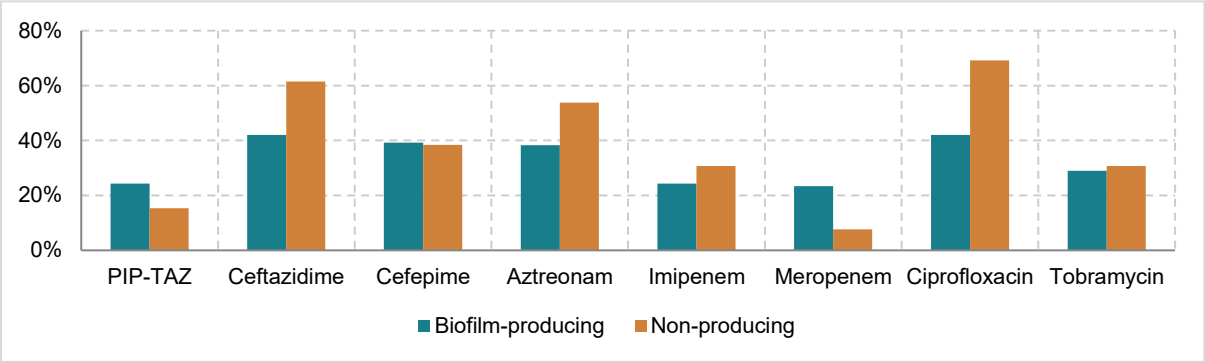


Figure 3. Antimicrobial Resistance Rates among Biofilm-Producing and Non-Biofilm-Producing Isolates.

4. Discussion

The data revealed high biofilm formation, primarily in weak and moderate phenotypes, and high carriage of matrix-associated and quorum-sensing genes. However, these illustrative patterns do not establish the prevalence or biological relationship between chronic wound infections and the clinical situation. Published investigations demonstrate the relevance of bacterial aggregates and organized bacterial aggregates in chronic wound studies, as they have been found directly at chronic wound sites [9]. Moreover, the dissemination of Pseudomonas aeruginosa aggregates, compared with that of Staphylococcus aureus, highlights that wound sampling affects the detection and interpretation of microbes [10]. In the present illustrative analysis, a positive relationship between the strength of the biofilm and the carriage of the gene was not observed and the estimated associations were large and encompassed the value of unity on their confidence intervals. Hence, the presence of a gene does not mean it is expressed. Experimental studies have shown that Pel cross-links extracellular DNA, and that it provides support for the biofilm matrix [11]. Similarly, Psl blocks complement-mediated opsonization and phagocytosis by neutrophils thus establishing a mechanistic connection between matrix deposition and immune escape [12]. The value of molecular characterization and the need for expression and functional assays are shown in such results. In the records, there was also no consistent relationship between antimicrobial resistance and biomass category. This observation does not support or refute experimental data showing that Psl protected against antibiotics during early biofilm development [13]. Importantly, planktonic susceptibility data for conventional susceptibility categories can match biofilm data, even though different physiological mechanisms may underlie biofilm tolerance. Borriello and coworkers showed that oxygen limitation plays a significant role in antibiotic tolerance in mature biofilms [14]. However, researchers recently discovered other persistence mechanisms beyond extracellular biomass in an intracellular bacterial aggregate remaining after antibiotic treatment in the zebrafish wound model [15]. The clinical relevance of these studies is that they provide information that could help investigate these microbes in combination with the host and the antimicrobial agents acting on them. The present simulation does not provide information for treatment but does provide

information. Isolate-level data, repeatable classification and corresponding phenotypic, molecular, and susceptibility summaries are available and are strong strengths of the analyses. The drawbacks of such synthetic observations are most significant, including completely synthetic observations, a small subpopulation of nonproducers, no data without cells, no gene-expression information, and no information relating to healing outcomes over time. In future clinical trials, these markers must be tested individually to demonstrate their ability to predict persistence or delayed healing in patients with affected wounds, using standardized tissue sampling, microscopy, quantitative expression analysis, validated biofilm susceptibility testing, and prospective wound assessment.

5. Conclusion

Biofilm and virulence mechanisms are useful tools for understanding Pseudomonas aeruginosa persistence in chronic wound infections. The analysis showed that carriage of algD, pelA, pslA, lasR, and rhlR, as well as biofilm-forming ability, varied. Gene carriage was not consistently associated with increased biofilm biomass, and the estimated associations were imprecise. No consistent trend of increased antimicrobial resistance emerged between biofilm groups, highlighting the need to evaluate antimicrobial resistance in planktonic and biofilm-associated cells separately. These observations are not meant to establish clinical prevalence, causal relationships or treatment recommendations, as the data were. They highlight an analytical approach that combines phenotypic biofilm assessment, molecular characterisation and antimicrobial susceptibility testing. Future studies of authentic patient specimens should include standard sampling methods, visual examination of wound biofilms, quantification of gene expression, and assessment of healing outcomes over time. These studies will help to answer the question if there is an independent association between biofilm-associated virulence markers and persistence of infection and whether virulence markers could contribute to improved treatment of chronic wounds.

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