Virulence factors as predictive tools for drug resistance in Pseudomonas aeruginosa

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P. aeruginosa is the fifth most frequent pathogen worldwide, the second in nosocomial pneumonia, the third in urinary infections, the forth in post-surgical infections and the seventh pathogen responsible for sepsis.1 It is also the major cause of mortality in cystic fibrosis patients and the most prevalent Gram-negative multidrug-resistant bacteria in the airway of mechanically ventilated patients and in pediatric patients hospitalized in intensive care units (ICU).1 Infections by P. aeruginosa are diverse and difficult to treat due to its intrinsic ability to develop resistance under antibiotic pressure and to produce a variety of virulence factors (VF), like adhesins, proteases, phenazines and exotoxins.3 Several studies report that the presence or expression of virulence traits is related to resistance to antibiotics.2,4,5,6 Some suggest these relations are antagonistic5,6 while others report enhanced resistance in isolates with high levels of VF.2,4 Also, literature indicates that inhibitors of specific VF may be good therapeutic alternatives to treat P. aeruginosa infections.7,8 With present work, we intend to address possible associations between VF and antibiotic resistance in P. aeruginosa, aiming to provide information that may be useful for the development of alternative therapies using VF inhibitors.

Seventy-six P. aeruginosa clinical isolates were randomly selected from sputum (43%), respiratory tract aspirates (21%), urine (20%), exudates (12%) and blood (4%) of inpatients and outpatients of a Portuguese central hospital, with consent from the patients. Antibiotic susceptibility tests were performed by disk diffusion method, using imipenem (IP), meropenem (MP), cefazidime (CAZ), ceftazidime (CAZ), piperacillin (PIP), amikacin (AMK) and ciprofloxacin (CIP). Results were interpreted according to CLSI recommendations,9 with intermediate results considered resistant. MDR classification was performed according to Magiorakos et al. (2011).10 Twelve virulence phenotypic characteristics were screened: swimming, swarmng, twitching motility and production of rhamnolipids,11 elastase,12 protease,13 lipase, lecithinase14 and pyocyanin.15 Production of phospholipase C, pyoverdine and biofilms were semi-quantified and classified as described elsewhere,16,17,18 considering 4 classes of production: absent, reduced, moderate and high. Screening for the genes flaG, orfF, pilA, pilB, associated to motility; lecA, lecB, involved in lectin production; apr, lasA, lasB, encoding protease and elastases; phzH, phzM, phzS, phzI, phzII, from the biosynthetic pathway of phenazines; exoA, encoding exotoxinA and exoS, exoT, exoU and exoY, encoding type III secretory system effector proteins, was carried out as previously described.3 exoA primers annealing temperature was 68°C with expected amplified fragments of 368 bp.19 Data were analyzed using SPSS® version 21.0 (IBM) software. Logistic regression (LR) modeling of antibiotic resistance as function of VF presence was performed retaining the predictors statistically significant in Wald test (p < 0.05). Models were selected according to the usual fit criteria: overall significance of the model (p < 0.05), Cox and Snell coefficient, Nagelkerke coefficient, Hosmer and Lemeshow test (p > 0.05) and the percentage of correctly classified cases.20 Although using the entire sample for LR analysis, we previously checked the isolates’ clonality by PFGE, as previously described3 and performed a preliminary LR analysis on the selected clones. With low robustness results provided by the used fit criteria, we tested the VF association to ATB by qui-square test (p < 0.05) of the clones and compared it with the entire sample. Since no differences were observed in the VF-ATB association results of the clones and the entire sample, we performed a final LR analysis with the entire sample, that returned more robust results, presented in Table 1.

The studied P. aeruginosa isolates showed high resistance levels, with 52.6% (n = 40) classified as multidrug-resistant.10 More than half of the population was resistant to IP, AZT, PIP and CIP (62.3%, 61%, 61% and 59.7%, respectively); 49.4% resisted to MP; 41.6% to CAZ and 40.3% to FEP. AMK was the antibiotic with better activity, with a resistance rate of 9.1%. Figures 1 and 2 present the prevalence of the tested virulence phenotypes and genes, respectively. All clinical isolates were able to swim and other binary phenotypes ranged from 84.4% (twitching) to 44.2% (pyocyanin) (Fig. 1A). 90.9% of all isolates presented high production of biofilms, while phospholipase C and pyoverdine reduced production were more common (Fig. 1B). All virulence genes were observed, except flaG (Fig. 2). exoT (89.6%) and phzI (80.5%) were the most prevalent genes and exoU (9.1%) and pilB (7.8%) the least. However, it is important to highlight that PCR-failure in protein variants is an acknowledged constraint of the technique. Multiple sets of
Table 1. Odds ratios for antibiotic resistance predicted by the presence of virulence factors (phenotypes or genes) in clinical Pseudomonas aeruginosa isolates

| ATB | VF | Level | p-valor (Wald) | Odds ratio | CI (95%) | %Corr.Clas | Over. Sig | R² (CS) | R² (N) | HL |
|-----|----|-------|----------------|------------|---------|-----------|------------|---------|---------|----|
| IP  | Pyover | 34vs12 | 0.010 | 8.912 | 1.706–46.545 | 81.3% | p < 0.001 | 0.429 | 0.588 | p = 0.801 |
|     | lecA | P vs A | 0.009 | 16.897 | 2.037–140.185 |
|     | lecB | P vs A | 0.003 | 0.018 | 0.001–0.262 |
|     | phzH | P vs A | 0.047 | 0.157 | 0.025–0.978 |
|     | exoY | P vs A | 0.005 | 0.147 | 0.006–3.398 |
| MP  | Pyover | 34vs12 | 0.001 | 47.657 | 4.421–513.762 | 80.6% | p < 0.001 | 0.431 | 0.575 | p = 0.880 |
|     | lecA | P vs A | 0.028 | 7.126 | 1.235–41.105 |
|     | lecB | P vs A | 0.001 | 0.072 | 0.015–0.352 |
|     | exoY | P vs A | 0.001 | 0.019 | 0.001–0.249 |
| CAZ | Pyover | 34vs12 | 0.012 | 10.533 | 1.694–65.482 | 83.6% | p < 0.001 | 0.537 | 0.721 | p = 0.780 |
|     | Rhamn | P vs A | 0.007 | 0.029 | 0.002–0.385 |
|     | Pyocya | P vs A | 0.013 | 13.490 | 1.746–104.235 |
|     | lecA | P vs A | 0.002 | 74.919 | 4.850–1157.367 |
|     | lecB | P vs A | 0.001 | 0.017 | 0.002–0.129 |
|     | exoT | P vs A | 0.017 | 44.173 | 1.963–994.218 |
| FEP | Pyover | 34vs12 | 0.002 | 8.708 | 2.231–33.988 | 80.8% | p < 0.001 | 0.405 | 0.546 | p = 0.295 |
|     | Rhamn | P vs A | 0.008 | 0.103 | 0.019–0.551 |
|     | Lipase | P vs A | 0.014 | 7.078 | 1.484–33.763 |
|     | lecB | P vs A | 0.001 | 0.074 | 0.016–0.343 |
| AZT | Pyover | 34vs12 | 0.010 | 11.441 | 1.791–73.071 | 77.0% | p < 0.001 | 0.408 | 0.558 | p = 0.512 |
|     | lecA | P vs A | 0.025 | 9.624 | 1.333–69.484 |
|     | lecB | P vs A | 0.002 | 0.017 | 0.001–0.218 |
|     | exoY | P vs A | 0.001 | 0.025 | 0.003–0.239 |
| PIP | Pyover | 34vs12 | 0.001 | 0.073 | 0.015–0.352 | 75.3% | p < 0.001 | 0.329 | 0.452 | p = 0.322 |
|     | Lipase | P vs A | 0.009 | 0.133 | 0.029–0.610 |
|     | PhosC | 4vs123 | 0.002 | 59.007 | 4.402–790.982 |
|     | exoA | P vs A | 0.001 | 0.054 | 0.009–0.318 |
| CIP | Pyover | 4 vs 2 | 0.013 | 7.407 | 1.526–35.714 | 78.4% | p < 0.001 | 0.310 | 0.422 | p = 0.596 |
|     | Rhamn | P vs A | 0.004 | 13.869 | 2.313–83.142 |
|     | PhosC | 4vs123 | 0.007 | 13.874 | 2.046–94.078 |
|     | phzM | P vs A | 0.012 | 0.135 | 0.029–0.639 |

Legend: Resistance predictor Susceptibility predictor

ATB – antibiotic; VF – virulence factor; CI (95%) – confidence intervals of odds ratio, with 95% significance level; % Corr. Clas. – percentage of cases correctly classified; Over. Sig. – overall significance; R² – proportion of explained variance; CS – Cox and Snell coefficient; N – Nagelkerke coefficient; HL – Hosmer and Lemeshow test; IP – imipenem; Pyover – pyoverdine; 1,2,3,4 – production classes (absent, reduced, moderate, high) of ordinal VFs (pyoverdine or phospholipase C); P – VF presence; A – VF absence; MP – meropenem; CAZ – ceftazidime; Rhamn – rhamnolipids; Pyocya – pyocyanin; FEP – ceftipime; AZT – aztreonam; PIP – piperacillin; PhosC – phospholipase C; CIP – ciprofloxacin.

Primers or DNA hybridization could have been used to confirm the negative results, but technical constraints impeded its application in this prospective study. Although PCR duplicate reactions of negative results, analysis of the DNA quality and quantity prior to the reactions and use of positive controls in every PCR-reaction were routinely performed during the study, some false negative results could still have occurred and are worth to explore in future similar studies.

Results from LR are presented in Table 1. AMK did not provide good regression models, due to its low resistance rate, being excluded from the study. Pyoverdine was the only VF present in all LR models, with its moderate or high production predicting the resistance to all antibiotics except PIP. Pyoverdine is a siderophore involved in iron acquisition, participate in biofilm formation and its chelating activity may be important in antibiotics resistance. A positive association between pyoverdine production and antibiotic resistance predictor

are required. Being both present in the cytoplasm and in the outer membrane of *P. aeruginosa*, apart from participating in biofilm formation, lectins also have different roles in bacterial virulence, with LecA more associated to cytotoxic effects and higher absorption of exotoxin A and LecB to pilus biogenesis and protease IV activity. These distinct virulence mechanisms presumably associated with distinct predictive drug resistances, as found in our study, deserve future attention as they may contain new pathways for drug targeting.

Rhamnolipids production, also involved in biofilm formation, was considered a good predictor for cephalosporins susceptibility and CIP resistance. The diminished expression of rhamnolipids in isolates with penicillinases and cephalosporinases has been reported, which concurs with our results. Regarding CIP resistance, one of the resistance mechanisms is the over-production of efflux pumps. Jeannot et al. refer rhamnolipids diminished expression in mutants with over-production of the efflux pump MexCD-OprJ, responsible for CIP efflux. In current study, presence of rhamnolipids was considered a good CIP resistance predictor, contradicting these authors. Other VF that intervened as antibiotic resistance predictors were only significant in few regression models (Table 1), for which their overall relevance is lower. Also, we must highlight that the majority of the tested virulence phenotypes and genes were not considered statistical significant predictors of resistance.

Overall, the observed predictive relation between VF genotypes or phenotypes and ATB resistance may suggest possible mechanistic relations between VF and ATB resistance that deserve being explored. It is relevant to emphasize that some VF associated to biofilm formation was considered good predictors of ATB resistance. Considering the fact that biofilm forms of pathogens are generally known to better resist antibiotherapy, the mechanisms involved in biofilm formation may have unexplored ATB resistance mechanisms that deserve higher attention in the future.
Technology to S. drug resistant strains, as those are the more concerning, due to the eminent lack of therapeutic options to treat the infections as new therapeutic strategies. These studies should focus mainly in multidrug resistant isolates, particularly extensively and pan-resistant efforts on new virulence inhibition drugs.

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Some authors refer that the use of compounds that inhibit or attenuate the action of specific VF is a good therapeutic alternative for the treatment of P. aeruginosa infections. However, this strategy can only be effective if the targeted VF is actually present in the bacterial cell, particularly in drug resistance strains. Otherwise the currently available antibiotics still remain as a good therapeutic option, as bacteria can be destroyed by them, leaving the new drugs to be used only when needed. This is a strategy of paramount importance to prevent development of resistance to new pharmaceutical chemicals. Regarding present results, suggesting a significant role of pyoverdin in P. aeruginosa resistance to several antibiotics, we think it is relevant to replicate similar studies in bigger samples of clinical populations of diverse origins to confirm this observation and provide further information for clinical practice and for the development of VF inhibitors as new therapeutic strategies. These studies should focus mainly in multidrug resistant isolates, particularly extensively and pan-drug resistant strains, as those are the more concerning, due to the eminent lack of therapeutic options to treat the infections they cause. Similar approach for other pathogens should also be performed, as it may also provide relevant information to direct research efforts on new virulence inhibition drugs.

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