Specific Type IV Pili groups in clinical isolates of *Pseudomonas aeruginosa*

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Abstract:

The relationships between specific Type IV Pili (TFP) groups and antibiotic resistance, biofilm formation and bacterial motility were determined in 190 *Pseudomonas aeruginosa* clinical isolates. While motility and biofilm formation were determined by phenotypic assays, the presence of TFP was determined by PCR assay and antibiotic susceptibility by disk diffusion. The results showed a high ability to form biofilm (97.4 %), multidrug resistance (44.7%) and the presence of a high number of motile isolates. We also found an association between strong biofilm production and multidrug resistance. Furthermore, TFP Group III was associated with strong biofilm production. On the other hand, the isolates with TFP Group II and those without any TFP were associated with non-strong biofilm production. Regarding motility, TFP Group II were associated with higher percentages of swarming, swimming and twitching, while TFP Group I showed lower percentages of swarming and twitching and TFP Group III showed lower levels of swarming and swimming. In conclusion, these findings highlight the differences in *P. aeruginosa* phenotypes related to the presence of specific TFP groups and their potential implications in clinical settings.

Keywords: bacterial motility; swarming; swimming; twitching; multidrug resistance; biofilm

Introduction

Pseudomonas aeruginosa is considered an opportunistic human pathogen being mainly associated with nosocomial infections. *P. aeruginosa* has the ability to rapidly develop resistance to antibiotics generating multidrug-resistant (MDR) isolates leading to serious problems in hospital settings (Moskowitz *et al.*, 2004).

P. aeruginosa populations undergo frequent recombination events contributing to the evolution of successful epidemic clones (López-Causapé *et al*., 2017). Thus, while diverse *P. aeruginosa* populations may be present in different hospital environments (Varin *et al*., 2017), well adapted clones may cause nosocomial outbreaks (Oliver *et al*., 2015).

A biofilm is defined as a community of microbial cells enclosed in an extracellular matrix and associated with a surface with architecture complexity (Deligianni *et al*., 2010). Despite containing molecules such as DNA or proteins, the extracellular matrix enclosing biofilm cells, is predominantly made up of different exopolysaccharides such as alginate, PEL or PSL (Mann and Wozniak, 2012). The specific relevance of each exopolysaccharide is related to specific bacteria characteristics (Høiby *et al*., 2017; Mann and Wozniak, 2012; Wozniak *et al*., 2003). Thus, alginate plays a mayor role in *P. aeruginosa* mucoid strains such as those prevalent in cystic fibrosis**,** and PEL and PSL are the most relevant amongst non-mucoid *P. aeruginosa* isolates (Høiby *et al*., 2017; Mann and Wozniak, 2012; Wozniak *et al*., 2003). Biofilm formation causes considerable problems in medical and industrial settings, because bacteria in biofilms may be resistant to antibiotic treatment, host immune responses, and biocide treatment (Harmsen *et al*., 2010).

Microscopic analyses have indicated that biofilm formation occurs in a sequential process of: (i) transportation of microbes to a surface; (ii) initial attachment; (iii) formation of microcolonies; (iv) biofilm maturation; and (v) biofilm dispersion (Klausen *et al*., 2003). *P. aeruginosa* presents three types of motility. Twitching motility, which is mediated by different factors including Type IV pili (TFP), allowing dissemination from the initial point of colonization via solid surfaces, interfaces or

moderate viscosities with repetitive alternating movements of extension and retraction (Kazmierczak *et al*., 2015). In addition, twitching is also involved in biofilm architecture and is responsible for the formation of microcolonies in biofilms (O'Toole and Kolter, 1998). Swarming and swimming motility are mediated by flagella and allow the movement of this microorganism on surfaces and in aqueous environments.

The major subunit of TFP is a protein encoded by the *pilA* gene. This gene is found at a conserved chromosomal locus between the adjacent *pilB* and $tRNA^{Thr}$ genes (Kus *et al*., 2004). In addition, *pilA* have five pili alleles with their accessory genes. Of these, only TFP group II presents the *pilA* gene without any accessory gene. TFP group I may be divided into subgroups defined by differences in the pili and accessory genes: Thus, subgroup Ia presents the $tfpO_a$ gene and Ib possesses the $tfpO_b$ gene (Kus et al., 2004). TFP group III and group V pilins possesses the *tfpY* and *tfpZ* accessory genes, respectively, and TFP group IV pili have the accessory genes *tfpW* and *tfpX*. The *pilA* alleles belonging to TFP group I ($pilA_{\mathrm{I}}$) and TFP group II ($pilA_{II}$) have shown to be more closely related among themselves than with TFP group III, IV and V pilins (Asikyan *et al*., 2008).

Differences in *P. aeruginosa* phenotypes related to the presence of a specific TFP group remain underexplored, being even less studied in clinical isolates of this microorganism. Therefore, the objective of this study was to determine the relationships between specific TFP Groups and antibiotic resistance, biofilm production and bacterial motility in *P. aeruginosa* isolates from patients from two hospitals in Lima, Peru.

Material and Methods

Bacterial isolates

A total of 190 *P. aeruginosa* isolates from clinical samples including bronchial secretions (sputum, trachea secretions and bronchoalveolar lavage), urine, wounds/abscesses and other (blood, body fluids, catheters and other unspecified sources) from patients attended at the Hospital Arzobispo Loayza (HAL, 78 strains) and the Hospital Nacional Cayetano Heredia (HNCH, 112 strains) from December 2012 to June 2013 were studied. In all cases only non-duplicated isolates from different patients were included in the study. The isolates were stored at -80ºC in skim milk medium until use. Each isolate was identified using conventional biochemical tests (Garcia, 2010). All samples were obtained within routine clinical practice; no personal data was requested or available to researchers.

Clonal Relationships

DNA fingerprinting of all isolates was generated by BOX-PCR as described previously (Mitov *et al*., 2010). In all cases the bacterial DNA was extracted by direct boiling (Feizabadi *et al*., 2010). The BOX-PCR profiles were analyzed according to the similarity of bands calculated by the Dice coefficient using Info Quest software (version 5) (Bio-Rad Laboratories, Inc). Parameters of 1.0% tolerance and 0.5% optimization were used, and similarity matrices were generated with the unweighted pair group method using arithmetic averages (UPGMA). Isolates showing ≥85 % of similarity were considered to be related.

Antimicrobial susceptibility determinations

Susceptibility to ceftazidime (CAZ, 30 µg), cefepime (FEP) (30 µg), aztreonam (ATM, 30 µg), imipenem (IMI, 10 µg), meropenem (MER) (10 µg), piperacillin-tazobactam (PTZ, 100/10 µg), gentamicin (GM, 10 µg), tobramycin (TO, 10µg), amikacin (AK, 10µg), ciprofloxacin (CIP, 5 µg),

126 levofloxacin (LVX, 5 µg), ofloxacin (OFX, 5 µg) and colistin (CO,10 µg) was established by the
127 disk diffusion test in Mueller - Hinton agar according to the methodology and guidelines proposed
128 by the Clinical and Laboratory Standards Institute (CLSI, 2017). The strain *P. aeruginosa* ATCC
129 22853 was used for quality control. Multidrug resistance was defined as resistance to three or more
130 unrelated antibiotics. Antibiotic non-susceptibility refers to the sum of intermediate and resistant
131 isolates.
132
133 *Biofilm growth assays*
134 Biofilms of each *P. aeruginosa* isolate were grown according to the methodology of Merritt *et al*
135 (2005). The optical density cut-off (ODc) value to separate biofilm-producer from non-biofilm-
136 producer isolates was calculated on the basis of three standard deviations (SD) above the mean
137 optical density (OD) of the negative control. Based on these OD values the isolates were classified
138 as follows: non biofilm producers [NBP] (OD ≤ ODc); weak biofilm producers [WBP] (ODc < OD
139 < 2 xODc); moderate biofilm producers [MBP] (2 xODc < OD < 4xODc); and strong biofilm
140 producers [SBP] (4xODc < OD) (Stepanovic *et al*., 2000).
141 The isolates were classified as SBP and non-SBP (MBP+WBP+NBP) for statistical purposes. The
142 reference strain *P. aeruginosa* PAO1 was used as a positive biofilm control.
143
144 *Motility Assays*
145 Swimming, swarming and twitching motilities were assayed on agar plates containing specific
146 medium according to the methodology of Gupta *et al.* (2016) and Deligianni *et al*. (2010). All the
147 plates were inoculated from an overnight culture using a sterile toothpick and were incubated at
148 37ºC for 48h (Deligianni *et al*., 2010; Gupta *et al*., 2016). Motility was determined as the radius of
149 the circular expansion of bacterial growth from the point of inoculation. For swimming and
150 swarming motility a measurable zone ≥25 mm was considered positive and twitching, was

151 considered as positive when ≥10 mm (Otton *et al*., 2017). The strain PAO1 was included in the
152 analysis as a positive motility control.
153
154 *TFP detection*
155 The presence of TFP was determined by PCR. The primers used (Table 1) were designed by Kus *et*
156 *al* (2004).
157
158 *Identification of accessory genes*
159 The primers used for identification of strains containing the *tfpO*$_a$, *tfpO*$_b$, *tfpW*, *tfpX*, *tfpY*, *tfpZ*
160 accessory genes downstream from *pilA* were designed for this study by our group (Table 1). The
161 PCR consisted of 15 min denaturation at 95 ºC followed by 30 cycles of 30s at 95 ºC, 1 min at
162 55ºC, 2 min at 72 ºC, with a final extension of 7 min at 72 ºC. The quality of the PCRs was
163 confirmed by the random selection of different amplified products to be sequenced.
164
165 *Statistical analysis*
166 The χ^2 (Chi square) test text was used to determine the presence of significant differences among
167 categorical data, which were considered statistically significant with a p value of ≤ 0.05.
168 Adjustments for multiple comparisons were made using the Holm and Benjamini–Hochberg
169 approaches.
170 The normal distribution of quantitative data set was established by the Shapiro-Wilk's W test; the
171 one-way ANOVA test and Tukey´s post hoc with 95% confidence interval was used to compare
172 differences between the individual TFP Groups. R study version 3.4.0. was used for all the
173 statistical analyses.

The associations of isolates possessing TFP group V were not analyzed because of the small number of positive isolates. When not explicitly indicated, TFP Groups Ia and Ib were analyzed together as TFP Group I.

Results

Bacterial isolates

During the study period, *P. aeruginosa* isolates were collected from a total of 190 non-duplicated isolates from different patients [Hospital Nacional Cayetano Heredia - HNCH (n=112); Hospital Arzobispo Loayza - HAL (n=78)]. *P. aeruginosa* was most frequently isolated from bronchial secretions 37.9% (72/190), urine 29.5% (56/190), and wounds/abscesses 17.4% (33/190). The proportion of isolates recovered from urine samples was significantly (p=0.0059) higher in HAL (32 isolates, representing 41% of HAL samples and 57.1% of total urine samples) compared to HNCH (24 isolates, representing 21.4% of HNCH samples and 42.9% of total urine samples). The colony characteristics of 187 isolates were similar to PAO1. Thus, none was mucoid, only three isolates showed morphology of small colony variant (SCV), all isolates but 2 were β-hemolytic, and 142 isolates were pigmented.

Clonal relationships

The analysis of the clonal relationships by BOX-PCR of the 190 isolates resulted in the identification of 72 clones (Supplementary Table). Of these, 27 (37.5%) were represented by a single isolate, 41 (56.9%) included 2 to 6 isolates and the remaining 4 clones included more than 6 isolates. In clones including more than one isolate, a high internal variability in terms of TFP groups, antibiotic resistance, biofilm production and bacterial motility was observed (Supplementary Table).

Antimicrobial resistance

High levels of non-susceptibility were observed among the 190 *P. aeruginosa* isolates, ranging from 38.4% (CAZ) to 56.3% (OFX), with similar results in both hospitals. Overall, 44.7% (85/190) were MDR, with no differences between the two hospitals studied (HNCH 44.6%, 50/112; HAL

44.9%, 35/78). (Table 2). In addition, the MDR isolates were also mainly from urine, 31/85 (36.5%), bronchial secretions 25/85 (29.4%) and wounds/ abscesses 13/85 (15.3%).

Biofilm production

Ninety-seven percent (185/190) of the isolates were biofilm producers. Of the total isolates, 41% (78/190) were SBP, 44.7% (85/190) were MBP, and 11.6% (22/190) and 2.6% (5/190) were WBP and NBP, respectively. Moreover, the SBP phenotype was significantly more frequent in the HNCH isolates [50.9% (57/112) vs. HAL 26.9% (21/78); p= 0.0009] (Fig.1).

In all cases, the SBP isolates exhibited higher levels of antimicrobial resistance than the non-SBP isolates, except in the case of PTZ in HAL. Overall, the SBP isolates were significantly more resistant to CAZ, FEP, ATM, IMI, MER, TO, AK and CIP (p<0.05), and accordingly, were associated with the presence of MDR (p=0.0179) (Table 2).When biofilm formation was examined according to the clinical origin of the samples, only isolates from wounds/abscesses [78.8% (26/33)] were associated with non SBP (p= 0.0186).

Motility phenotype

Twitching, swarming and swimming motilities were analyzed in 189 isolates recovered from frozen stock. Of these, 86.2% (163/189) presented twitching, while 57.7% (109/189) and 83.1% (157/189) showed swarming and swimming, respectively. MDR isolates presented rates of positive migration zones of 83.5% (71/85), 40% (34/85) and 76.5% (65/85) for twitching, swarming and swimming motilities, respectively, and non-MDR isolates showed rates of 88.5% (92/104), 72.1% (75/104), 88.5% (92/104), respectively. The results showed that non-MDR isolates were significantly associated with swarming and swimming motility (p<0.0001 and p=0.0287), respectively.

Type IV pili

Overall, 161/189 (85.2%) of the isolates presented at least one TFP. Of these, 135 (83.9%) presented only one TFP group, and the remaining 26 (16.1%) had more than one TFP group (Tables 3 and 4). TFP Group II was the most frequently detected, being found in a total of 103 isolates; being the only TFP group detected in 83 of these isolates (43.9% of the total isolates, 51.5% of isolates possessing TFP). TFP Group I was detected in 45 isolates (together with other TFPs in 16 isolates). Group III was detected in 34 isolates (together other TFPs in 14 isolates). Group V was present in 12 isolates, being the only TFP in 3 isolates. No isolate carrying the TFP Group IV was detected (Table 4).

The absence of TFP (p=0.0005) as well as the presence of only the TFP Group II (p=0.0260) was significantly higher in the isolates from HAL, while the presence of TFP Group I (p=0.0153) and Group III (p=0.0260) was higher among HNCH isolates. Isolates carrying more than one TFP were also more frequent in HNCH (p=0.0044).

TFP and multiresistance

Analysis of the association between TFP and multidrug resistance showed that 17/26 (65.4%) isolates with multiple TFP, 57/135 (42.2%) isolates with only one TFP and 11/28 (39.3%) of those without TFP were MDR. Nonetheless, analysis by TFP groups showed that the presence of only TFP Group II (25/83 isolates, 30.1%) was associated with the presence of a non-MDR phenotype (p=0.0014), while the percentages of multidrug resistance among isolates belonging to Groups I and III were 58.6% and 70.0%, respectively (Table 4, Fig 2a).

TFP, motility and biofilm

Overall, 14.9% (24/161) of the isolates presenting TFP did not show twitching motility, while 92.9% (26/28) of the isolates in which no TFP group was detected did.

On evaluating the relationship between the TFP group and flagellar motility (swarming or swimming) it was observed: the presence of only TFP Groups I and III were associated with less swarming (p=0.023/p=0.024); TFP Group III also showed less swimming motility (p=0.022), and isolates possessing only TFP group I were those with the least twitching motility (p=0.045) (Fig 2b, Table 4). The highest levels of twitching were found among the isolates with TFP Group II and those without TFP (>90% in both cases).

Overall, the presence of TFP was associated with SBP (p=0.02), but when biofilm formation was related to the different TFP groups, only Group III showed a significant association with SBP (p=0.025), while Group II (p=0.025) was associated with non-SBP and those without TFP almost reached significance (Fig. 2c, Table 4). Similarly, the one-way ANOVA test showed a p=0.058, bordering the significance breakpoint, when TFP contribution to biofilm biomass was determined (Fig. 3a). On analyzing the relationship between TFP groups and biofilm biomass with the one-way ANOVA test a significant association was observed (p<0.0001) with Groups I, III and those with more than one TFP presenting greater biofilm biomass (Fig. 3b).

Swarming motility was associated with non-SBP (p<0.0001) and lower biofilm biomass (p<0.0001) (Fig. 3c). Similarly the presence of swimming tended to be related to non-SBP (p=0.058) and significantly associated with lesser biofilm biomass (p<0.0001) (Fig. 3e).

On the other hand, twitching motility was also associated with non-SBP (p=0.0072) and lower biofilm biomass (p<0.0001) (Fig 3g). On analyzing the specific role of each TFP Group, irrespective of the type of motility, isolates presenting TFP Groups I and III and those with more than one TFP presented greater biofilm biomass, while those with TFP Group II or without TFP presented lower biofilm biomass levels, (Figs. 3d, 3f, 3h). Finally, those isolates without swarming, swimming or twitching showed higher levels of biofilm biomass (Fig. 4).

Discussion

This study was aimed to determine the relationships between specific TFP groups and antibiotic resistance, biofilm production and bacterial motility. The prevalence of the different TFP groups observed in the present study are similar to those of other authors in which TFP Group I was present in 18% (28/159) of human clinical isolates and TFP Group III represented 7% of 244 isolates of *P. aeruginosa* obtained from a wide range of environments (Kus *et al*., 2004; Asikyan *et al*., 2008). To our knowledge, no specific study has determined the presence of possible associations of specific TFPs with motility, enhanced biofilm formation or factors favoring the selection of isolates carrying multiple TFP.

Our results showed a relation between the number of TFP groups and multidrug resistance. Thus, isolates without TFP showed the lowest levels of multidrug resistance. Nonetheless, although the reason for this is not clear, around 60% of isolates carrying TFP Group I or III were MDR while isolates having the TFP Group II were more prone to be non-MDR. Further analyses in clinical settings from both Peru and other countries are needed to demonstrate this association providing a rationale to determine the underlying reasons.

The implication of *pilA* in swarming motility needs to be fully elucidated. Thus, while Shrout *et al*., (2006) showed swarming motility was not impaired in a *pilA* deficient strain and, in some circumstances even showed hyperswarming motility, Köhler *et al*., (2000) observed the absence of swarming in TFP-deficient *P. aeruginosa* mutants. The present study showed differences in the association of the TFP groups with swarming and also swimming phenotypes. In agreement with the association of swimming and swarming with flagellar activity, the presence of TFP in our study was not correlated with these motilities, with motile and non-motile isolates presenting TFP. Nonetheless, our data show that the presence of TFP Groups I and III is related to a lower presence of swarming and swimming ability. This result provides additional information to the previous description of the impairment of swarming motility related to the presence of TFP (Anyan *et al*.,

2004) demonstrating the association of this finding with specific TFP groups. Interestingly, twitching motility was also more frequently present among isolates with TFP Group II and among those without TFP. Thus, in our study 92.8% (26/28) of the isolates not associated with any TFP group were twitching phenotype positive. While the presence of other adhesins cannot be ruled out (Chiang and Burrows, 2003**)**, the presence of polymorphisms on the primers annealing region, and that of undescribed TFP possessing new arrangements or longer additional complementary genes leading to long DNA regions which are difficult to amplify with the present PCR conditions and reagents should also be considered. Similarly, the possible insertion of a mobile element before or after TFP genes, which does not affect the functionality of the TFP but does impair PCR amplification, is another possible explanation. On the other hand, isolates possessing TFP but without twitching motility may be explained by the presence of altered or inactive TFP or by the lack of or underexpression of other necessary genes (Chiang and Burrows, 2003).

This study suggests an association between specific TFP and levels of biofilm formation. In this sense, it was of note that the presence of TFP was significantly associated with SBP, and a *p* value of 0.058 was observed when the presence of TFP was associated with biomass levels. In this way, the biofilm formation is classified by categories, while biomass is a numerical data obtained from the OD which explains this slight difference. Several studies have reported the importance of bacterial motility to initiate contact with an abiotic surface, biofilm formation and development (Deligianni *et al*., 2010; O'Toole *et al*., 2000). Nonetheless, the associations between the presence of specific TFP groups and biofilm formation ability remain understudied, although they might be related to the absence or presence of TFP accessory genes. In this sense, although it has been shown that a higher twitching level impairs the ability of biofilm formation (Haley *et al*., 2014), it has been suggested that twitching is one of the factors involved in the first stages of biofilm formation (O'Toole *et al*., 2000), and consequently, in the degree of biofilm biomass (Deligianni *et al*., 2010). Furthermore, the presence of TFP accessory genes may be correlated with enhanced

329 twitching (Asikyan *et al*., 2008). Therefore, the presence of accessory genes might positively
330 influence the biofilm formation. Our results agree with the impairment of biofilm formation related
331 to the presence of twitching (Haley *et al*., 2014) and isolates presenting twitching are correlated
332 with lower levels of biofilm biomass. Furthermore, the higher levels of biomass biofilms were
333 correlated with the absence of swimming, swarming and twitching, while isolates presenting the
334 three types of motility were those with lower biofilm biomass. This result highlights the fact that
335 isolates with lower motility levels are more prone to adhere to surfaces leading to biofilm
336 formation. Nevertheless, contrary to the proposed correlation between TFP accessory genes and
337 enhanced twitching (Asikyan *et al*., 2008), in all the isolates presenting TFP Groups II, the only
338 TFP Group lacking accessory genes, or without any TFP showed higher levels of motility.
339 On the other hand, the presence of isolates without TFP classified as SBP and that of isolates with
340 a very poor adhesion to the microtiter plate despite possessing TFP (including TFP Group III)
341 agree with the fact that mutants deficient in pili and flagella do not exhibit significant differences
342 regarding biofilm formation compared to wild type strains (Klausen *et al*., 2003). Therefore, these
343 findings point to the possible role of other new adhesins that could participate in the initial stage of
344 biofilm formation (Otton *et al*., 2017; Head and Yu, 2004), and highlight the multifactorial nature
345 of biofilm formation.
346 In a recent study the development of quinolone resistance was correlated with lower levels of
347 twitching activity (Ahmed *et al*., 2018). Thus, as our results showed that isolates presenting lower
348 motility levels were related to SBP, this finding may be related to the high levels of antibiotic
349 resistance detected in those isolates being SBP. Accordingly, all isolates deficient for the three
350 tested motilities were SBP and MDR, and the presence of multidrug resistance was lesser among
351 those isolates possessing TFP Group II or those without TFP which showed high motility levels.
352 Our results showed the presence of differences in the prevalence of TFP as well as of TFP groups
353 and other characteristics among the samples from the two hospitals. This finding suggests the

presence of differences among both bacterial populations. Nonetheless, no specific reason may be adduced.

The intraclonal variability of the parameters analyzed must be considered among limitations of this study, since it prevents analysis corrected by phylogeny. This variability may be related to horizontal gain or loss of genetic material, or with undetected punctual mutations, insertions or deletions which affect the final expression levels of key genes.

Overall, the presence of TFP was correlated with enhanced biofilm formation; furthermore, the presence of specific TFP Groups was correlated with different findings, including MDR (Group III) /non-MDR (Group II) profiles and levels of swarming / swimming / twitching motility (Group I and Group III with lower levels of motility, Group II or those without TFP with higher levels of motility). The present results suggest that isolates with impaired motility are more prone to being SBP. These findings highlight the need for an in-depth analysis of the underlying differences and elucidation of the exact relationships between specific TFP groups, biofilm production and the acquisition of multidrug resistance.

368 **Compliance with ethical standards**

369

370 **Conflict of interest**:

371 The authors declare that they have no conflict of interest.

372

373 **Ethical statement**

374 The study was approved by the Ethical Committee of the Universidad Peruana Cayetano Heredia

375 (Lima, Peru) and by the Ethical Committee of Hospital Clinic (Barcelona, Spain).

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Legends to Figures

Fig 1: Biofilm formation and multidrug resistance

HNCH: Hospital Nacional Cayetano Heredia; HAL: Hospital Arzobispo Loayza. SBP: Strong biofilm producer; MBP: Moderate biofilm producer; WBP: Weakly biofilm producer; NBP: non biofilm producer.

* p=0.0009

Fig 2 Distribution of TFP groups according to multidrug resistance, biofilm formation and bacterial motility.

TFP: Type 4 Pili; MIX Group: isolates possessing more than one group of TFP; None group: Isolates without any TFP group.

MDR: Multidrug resistance; NBP: Non biofilm producers; WBP: Weak biofilm producers; MBP: Moderate biofilm producers; SBP: Strong biofilm producers;

Fig 2a: TFP groups according to MDR and non-MDR isolates.

Fig 2b: TFP groups according to motility.

Fig 2c: TFP groups according biofilm formation.

Fig 3. Box plots showing the association of TFP, swarming or swimming with biofilm biomass.

For swimming and swarming motility a measurable zone ≥25 mm was considered positive and twitching, was considered as positive when ≥10 mm (Otton *et al*., 2017).

Fig. 3a: TFP and biofilm biomass (p = 0.058).

Fig. 3b: Association of each TFP group with biofilm biomass (p< 0.0001).

Fig. 3c: Association of swarming motility with biofilm biomass (p < 0.0001).

Fig. 3d: Presence (+) / absence (-) of swarming according to TFP groups and biofilm biomass ($p <$ 0.0001).

Fig. 3e: Association of swimming motility with biofilm biomass

Fig. 3f: Presence (+)/ absence (-) of swimming according to TFP groups and biofilm biomass ($p< 0.0001$).

Fig 3g: Association of twitching motility with biofilm biomass ($p<0.0001$)

Fig 3h: Presence (+)/ absence (-) of twitching according to TFP groups and biofilm biomass ($p< 0.0001$).

None: represents those isolates without any TFP; mix: shows isolates with more than one TFP. CV: Crystal Violet. Cercles (°): outliers showing values of biofilm biomass get out range.

Fig 4 **Box plots showing the association of motility with biofilm biomass.**

For swimming and swarming motility a measurable zone ≥25 mm was considered positive and twitching, was considered as positive when ≥10 mm (Otton *et al*., 2017).

Absence: Lack of swarming, swimming and twitching; Presence: Concomitant presence of swarming, swimming and twitching; Mixture: Presence of a minimum of one motility type and a maximum of two motility types. When the different combinations of "Mixture" motility group were analyzed individually no differences were observed among them.

Table 1. Primers used in this study.

Amplified product	Primer	Sequence (5' → 3')	Amplicon size (bp)	Reference
TFP and accessory genes				
TFP	pilB tARN^{Thr}	TCC AGC AGC ATC TTG TTG ACG AA CGA ATG AGC TGC TCT ACC GAC AGA GCT	Group Ia (2821) Group Ib (2797) Group II (1370) Group III (2185) Group IV (4452) Group V (2289)	Kus *et al*., 2004
tfpO$_a$	tfpO$_a$ - F tfpO$_a$ - R	TCT ATT ATT GCT GAT AAG TAT TC GCC AAT ACG GTC TGG GTG AA	1113	This study
tfpO$_b$	tfpO$_b$ - F tfpO$_b$ - R	CAC TGC TAT TCC TGA TAG CAG GAA ATA GAG CGC CAG TCC GA	713	
tfpW	tfpW - F tfpW - R	TGC TCT GCC TAT GTA TGG CG CAA GGA ATG CTA AGG GGG CA	582	
tfpX	tfpX - F tfpX - R	GGG AAA ATG GTA TCC GCC CC CTC CGG AGG CGA ACT CTA CT	314	
tfpY	tfpY - F tfpY - R	TAG TGC GTG ACT TGG GTG TC CCA ATT GGG TCT GTA GCG GT	288	
tfpZ	tfp Z - F tfp Z - R	ATT AGG GCG TTC GCT GTT CA GGT ACC TAC CAA CTG CCA CC	594	
Clonal relationships				
BOX-PCR		CTA CGG CAA GGC GAC GCT GAC G	Variable	Mitov *et al*., 2010

TFP: Type IV pili; F: Forward; R: Reverse; bp: base pair

Table 2: Antimicrobial resistance (%) of *Pseudomonas aeruginosa* isolates according to the strong biofilm producer phenotype.

Antimicrobial	Total (n=190)					HNCH(n=112)					HAL(n=78)				
	Overall	SBP (n=78)	Non-SBP † (n=112)	*p* value	*p* adjusted	Overall	SBP (n=57)	Non-SBP † (n=55)	*p* value	*p* adjusted	Overall	SBP (n=21)	Non-SBP † (n=57)	*p* value	*p* adjusted
CAZ	38.4	50.0	30.4	**0.006**	**0.0179**	39.3	49.1	29.1	**0.03**	0.0754	37.2	52.4	31.6	0.091	0.2123
FEP	42.6	53.8	34.8	**0.009**	**0.0195**	43.8	54.4	32.7	**0.02**	0.0754	41.0	52.4	36.8	0.215	0.2541
ATM	45.8	55.1	39.3	**0.031**	**0.0491**	49.1	56.1	41.8	0.129	0.1524	41.0	52.4	36.8	0.215	0.2541
IMI	50.5	62.8	42.0	**0.005**	**0.0179**	53.6	63.2	43.6	**0.038**	0.0754	46.2	61.9	40.4	0.090	0.2123
MER	46.3	59.0	37.5	**0.003**	**0.0179**	47.3	56.1	38.2	0.057	0.0754	44.9	66.7	36.8	**0.018**	0.2123
GM	46.8	55.1	41.1	0.057	0.0741	43.8	52.6	34.5	0.053	0.0754	51.3	61.9	47.4	0.254	0.2752
TO	46.8	56.4	40.2	**0.027**	**0.0491**	43.8	52.6	34.5	0.053	0.0754	51.3	66.7	45.6	0.098	0.2123
AK	42.1	53.8	33.9	**0.006**	**0.0179**	41.1	50.9	30.9	**0.031**	0.0754	43.6	61.9	36.8	**0.047**	0.2123
PTZ	34.7	42.3	29.5	0.067	0.0792	38.4	49.1	27.3	**0.017**	0.0754	29.5	23.8	31.6	0.504	0.5040
CIP	51.1	60.3	44.6	**0.034**	0.**0491**	49.1	57.9	40.0	0.058	0.0754	53.8	66.7	49.1	0.168	0.2541
LVX	53.2	60.3	48.2	0.101	0.1094	51.8	57.9	45.5	0.187	0.2026	55.1	66.7	50.9	0.213	0.2541
OFX	56.3	62.8	51.8	0.131	0.1310	55.4	59.6	50.9	0.352	0.3520	57.7	71.4	52.6	0.136	0.2526
MDR	44.7	56.4	36.7	**0.0069**	**0.0179**	44.6	54.3	34.5	**0.0347**	0.0754	44.9	61.9	38.6	0.064	0.2123

HNCH: Hospital Nacional Cayetano Heredia; HAL: Hospital Arzobispo Loayza; CAZ: Ceftazidime, FEP: Cefepime, ATM: Aztreonam, IMI: Imipenem, MER: Meropenem, GM: Gentamicin, TO: Tobramicin, AK: Amikacin, PTZ: Piperacillin/Tazobactam, CIP: Ciprofloxacin, LVX: Levofloxacin, OFX: Ofloxacin. MDR: Multidrug resistant

SBP: Strong biofilm producers.

† “Non-SBP” represents MBP (moderate biofilm producers) + WBP (weak biofilm producers) + NBP (non-biofilm producers) isolates.

p: significant differences between SBP vs. non-SBP.

All isolates were susceptible to colistin.

Table 3. Distribution of TFP groups in MDR and non-MDR isolates.

MDR isolates		Non-MDR isolates	
TFP groups	n = 85	TFP groups	n = 104
Ia	14	Ia	6
Ib	2	Ib	1
II	25	II	58
III	14	III	6
V	1	V	2
Ia - Ib	1	Ia - Ib	5
Ia - II	1	Ia - II	2
Ia - III	3	Ia - V	1
II - III	4	II - V	1
II - V	1	III - V	1
III - V	1	Ia - Ib - II	2
Ia - Ib -II	2	Ia - II - III	1
Ia - II - III	1	II - III - V	1
Ia - II - V	1	none	17
II - III - V	1		
Ia - Ib - II - III	1		
Ia - Ib - II - V	1		
none	11		

TFP: Type IV Pili; MDR: Multidrug resistant

Table 4. Distribution of pilin alleles among analyzed isolates

Pilin allele	**No**		**I**	**II**	**III**	**IV**	**V**	**More than one TFP group**	**Not TFP**
Accesory gene (s)			*tfpO*	None	*tfpY*	*tfpWX*	*tfpZ*	Mixture	---
Number of isolates	189		29 (15.3 %)	83 (43.9%)	20 (10.6%)	0 (0.0%)	3 (1.6%)	26 (13.7%)	28 (14.8%)
HNCH	112		24 (82.8 %)	41 (49.4 %)	17 (85.0%)	0 (0.0%)	3 (100%)	23 (88.4%)	4 (14.3%)
HAL	77		5 (17.2%)	42 (50.6%)	3 (15.0%)	0 (0.0%)	0 (0.0%)	3 (11.5%)	24 (85.7%)
HNCH vs HAL		***P value***	**0.0051**	**0.0146**	**0.0132**	ND	ND	**0.0011**	**<0.0001**
		P adjusted	**0.0153**	**0.0260**	**0.0260**	ND	ND	**0.0044**	**0.0005**
Bonchial secretions	72		14 (48.3%)	31 (37.3%)	7 (35.0%)	0 (0.0%)	2 (66.6%)	9 (34.6%)	9 (32.1%)
		P value	0.2198	0.8518	0.76308	ND	ND	0.6940	0.4822
Wounds/Abcesses	33		4 (13.8%)	15 (18.0%)	3 (15.0%)	0 (0.0%)	0 (0.0%)	4 (15.4%)	7 (25.0%)
		P value	0.5718	0.8445	0.7592	ND	ND	0.7640	0.2548
Urine	55		6 (20.7%)	26 (31.3%)	4 (20.0%)	0 (0.0%)	1 (33.3%)	8 (30.8%)	10 (35.7%)
		P value	0.278	0.551	0.343	ND	ND	0.840	0.403
Other	29		5 (17.2 %)	11 (13.3%)	6 (30.0%)	0 (0.0%)	0 (0.0%)	5 (19.2%)	2 (7.1%)
		P value	0.7580	0.4803	0.0545	ND	ND	0.5538	0.1920
MDR	85		17 (58.6%)	25 (30.1%)	14 (70.0%)	0 (0.0%)	1 (33.3%)	17(65.4%)	11 (39.3%)
		P value	0.1084	**0.0003**	**0.0173**	ND	ND	**0.024**	0.512
		P adjusted	0.260	**0.0014**	0.0680	ND	ND	0.0720	0.520
SBP	78		16 (55.1%)	25 (30.1%)	14 (70.0%)	0 (0.0%)	2 (66.6%)	15 (57.6%)	6 (21.4%)
		P value	0.098	**0.0059**	**0.0058**	ND	ND	0.067	**0.0209**
		P adjusted	0.134	**0.025**	**0.025**	ND	ND	0.134	0.063
Twitching positive	163		21 (72.4%)	78 (94.0%)	16 (80.0%)	0 (0.0%)	1 (33.3%)	21 (80.8%)	26 (92.9%)
		P value	**0.0188**	**0.0063**	0.3913	ND	ND	0.3829	0.2710
		P adjusted	**0.045**	**0.023**	0.491	ND	ND	0.491	0.450
Swarming positive	109		10 (34.5%)	58 (69.9%)	6 (30.0%)	0 (0.0%)	2 (66.6%)	13 (50.0%)	20 (71.4%)
		P value	**0.006**	**0.0026**	**0.0081**	ND	ND	0.3939	0.1104
		P adjusted	**0.023**	**0.022**	**0.024**	ND	ND	0.491	0.206
Swimming positive	157		23 (79.3%)	74 (89.2%)	12 (60%)	0 (0.0%)	3 (100%)	21 (80.7%)	24 (85.7%)
		P value	0 .5575	**0.0483**	**0.0036**	ND	ND	0.7364	0.6859
		P adjusted	0.642	0.102	**0.022**	ND	ND	0.736	0.733

No: Number; TFP: Type IV Pili; I: TFP Group I, II: TFP Group II; III: TFP Group III; IV: TFP Group IV; V: TFP Group V; None: absence of accessory gene; More than one TFP group: concomitant presence of different TFPs in the same isolate; Not TFP: Absence of TFP; HNCH: Hospital Nacional Cayetano Heredia; HAL: Hospital Arzobispo Loayza, MDR: Multidrug Resistant; SBP: Strong Biofilm Producer

Significant differences in bold and highlighted in grey.

Figure 1

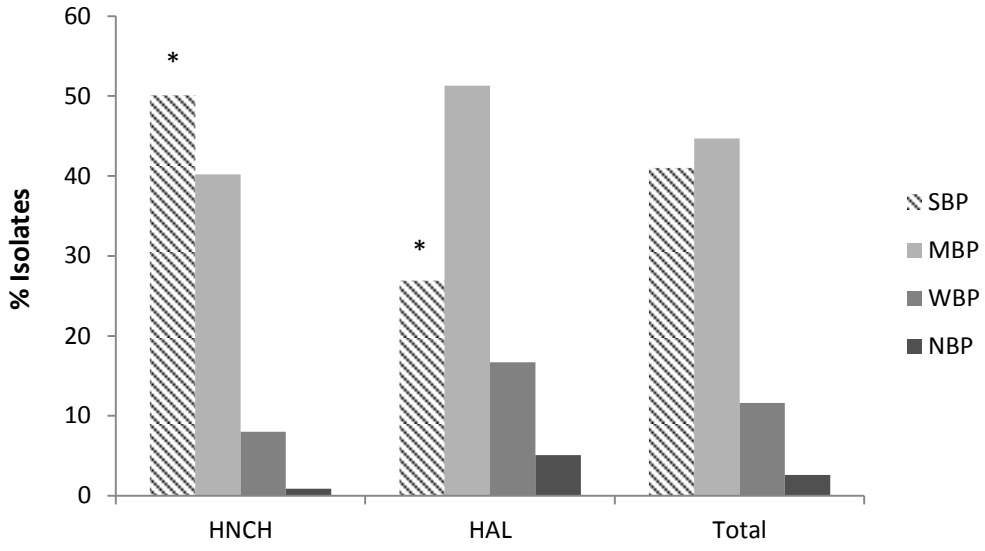
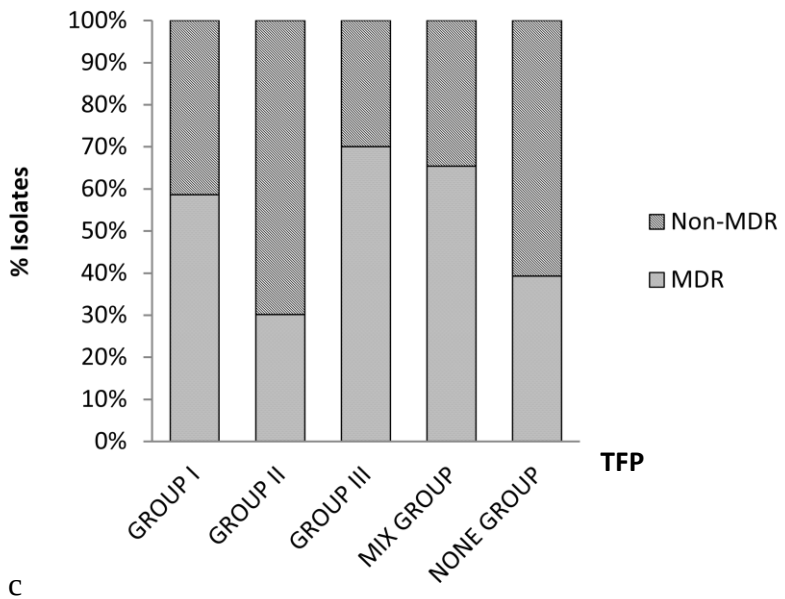
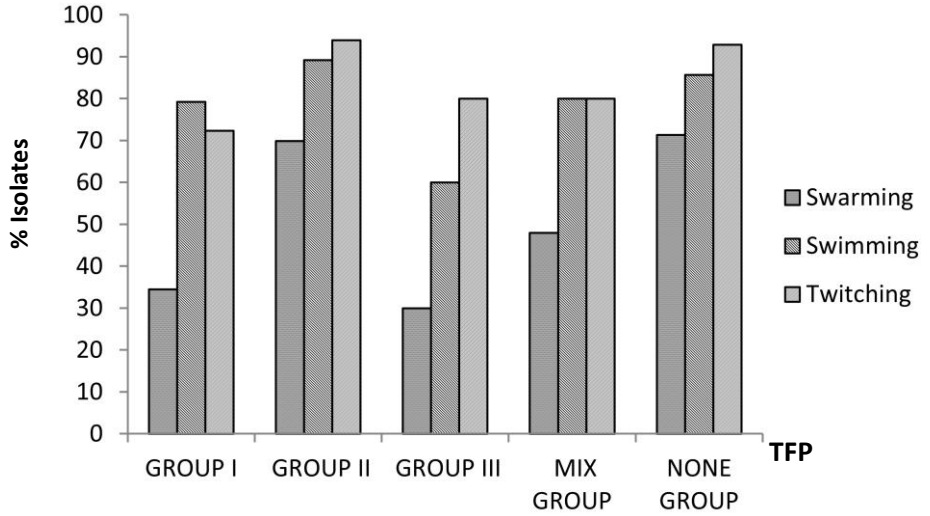


Figure 2

a


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% Isolates
GROUP I
GROUP II
GROUP III
MIX GROUP
NONE GROUP
TFP
Non-MDR
MDR

b


100
90
80
70
60
50
40
30
20
10
0
% Isolates
GROUP I
GROUP II
GROUP III
MIX GROUP
NONE GROUP
TFP
Swarming
Swimming
Twitching

c

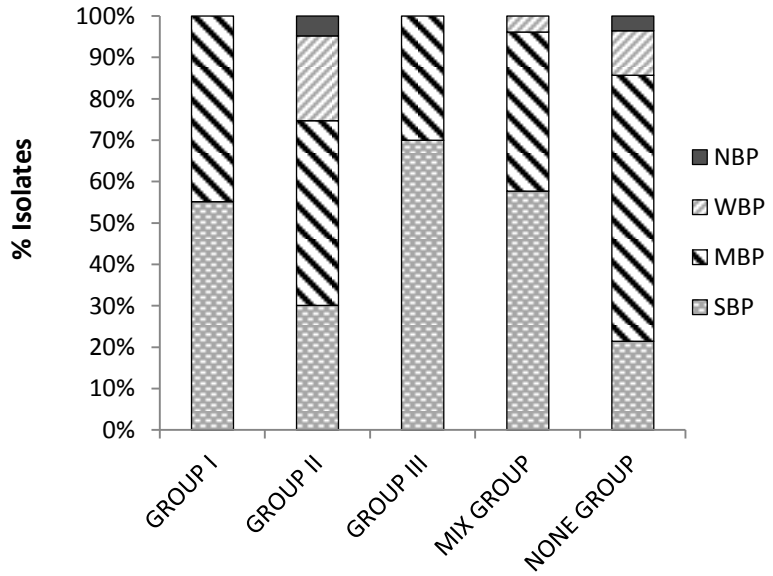
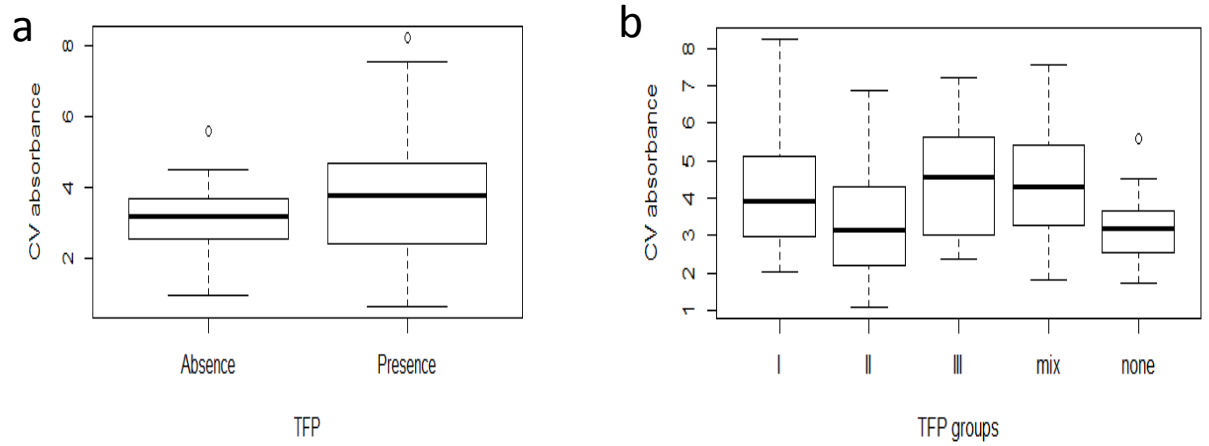
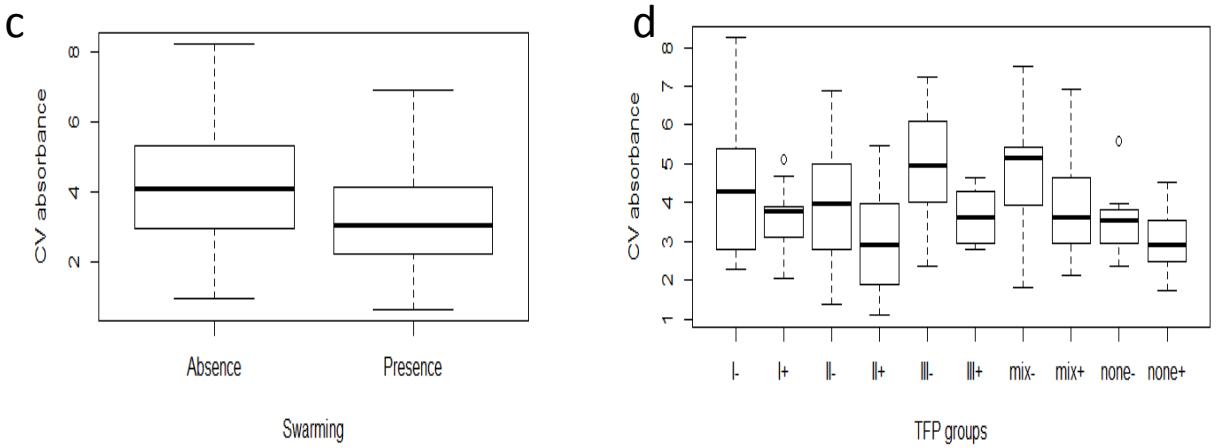

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% Isolates
GROUP I
GROUP II
GROUP III
MIX GROUP
NONE GROUP
NBP
WBP
MBP
SBP

Figure 3

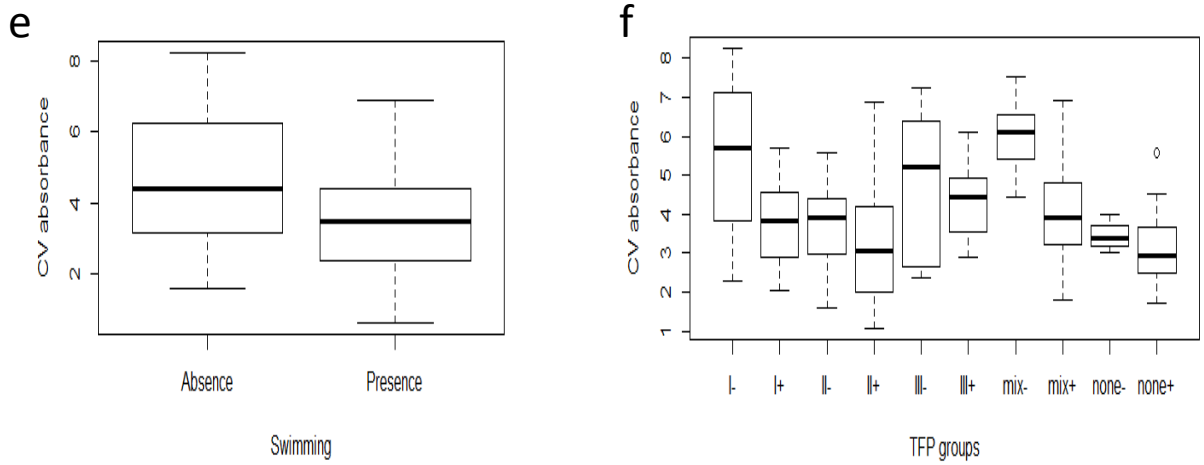
Type IV Pili



Swarming



Swimming



Tiwtching

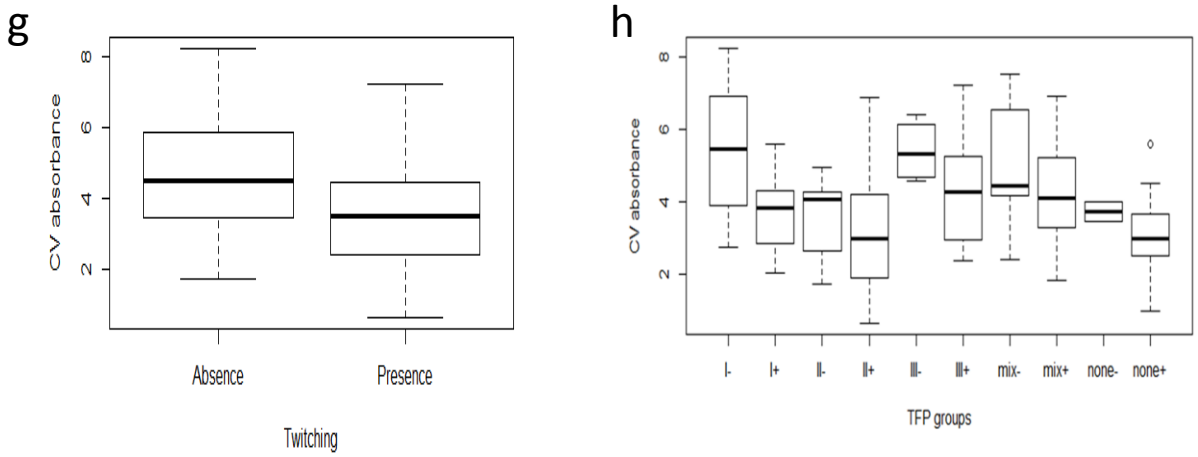
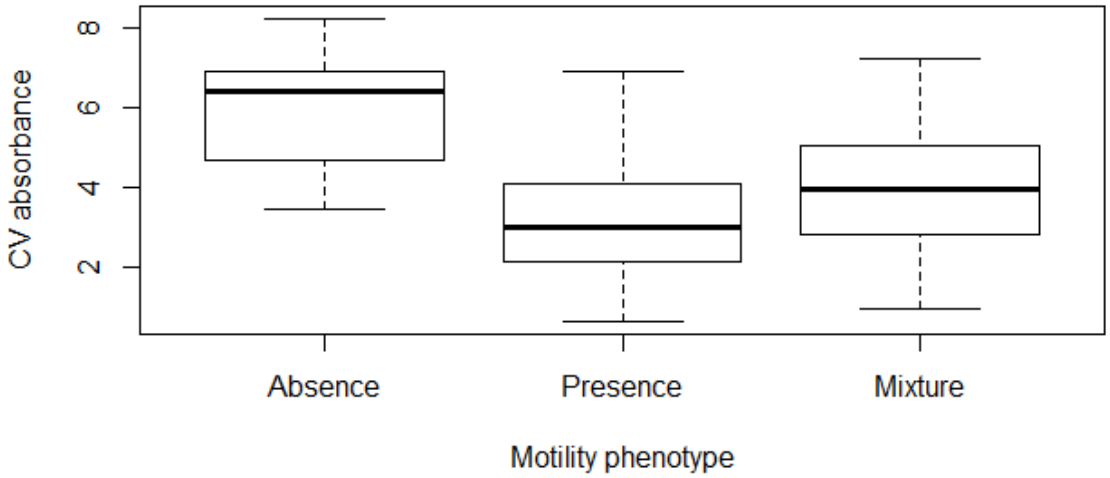


Figure 4


CV absorbance
2
4
6
8
Absence
Presence
Mixture
Motility phenotype

Genotypes	Total no. isolates	Hospital A/B	Kind of samples	Pattern of Resistance (no. of isolates)	Biofilm Producing	TFP groups	Motility		
							Twitching	Swarming	Swimming
			Catheter	CAZ,FEP,ATM,IMI,MER,GM,TO,AK,CIP,LVX,OFX,PTZ		none	-	-	-
1	3	A	Bronchial Secretion	-	2	Ia	+	-	-
			Bronchial Secretion	-		none	-	-	-
2	1	A	Sputum	-	1	III	-	+	-
3	1	A	Tracheal secretion	-	2	II	+	-	-
			Sputum	CAZ,FEP,ATM,IMI,MER,GM,TO,AK,CIP,LVX,OFX,PTZ	2	III	+	-	-
			Feces	CAZ,FEP,ATM,IMI,MER,GM,TO,AK,CIP,LVX,OFX,PTZ	1	II - III	+	-	-
4	5	A	Feces	CAZ,FEP,ATM,IMI,MER,GM,TO,AK,CIP,LVX,OFX,PTZ	1	III	+	-	+
			Wounds/abscess	CAZ,FEP,ATM,IMI,MER,GM,TO,AK,CIP,LVX,OFX,PTZ	1	III	+	-	+
			Wounds/abscess	-	2	Ia	+	+	+
5	1	A	Feces	-	2	none	+	+	-
		A	Urine	CAZ,FEP,ATM,MER,GM,TO,AK,CIP,LVX,OFX	2	II	+	+	+
		A	Bronchial secretion	-	2	II - III - V	-	+	+
		B	Urine	CAZ,FEP,ATM,IMI,MER,GM,TO,AK,CIP,LVX,OFX	1	none	+	+	+
6	7	B	Wounds/abscess	ATM,IMI,MER,GM,TO,AK,CIP,LVX,OFX,PTZ	1	II	+	+	+
		B	Urine	CAZ,FEP,ATM,IMI,MER,GM,TO,CIP,LVX,OFX	1	II	+	+	+
		B	Urine	-	2	II	+	+	+
		B	Urine	-	3	ND	ND*	ND	ND
		A	Tracheal secretion			Ia	+	-	+
7	3	B	Urine	-	2	Ib	+	+	+
		B	Tracheal secretion			none	+	+	+
8	1	B	Sputum	-	2	II - Ia	+	+	+
			Urine	CAZ,FEP,ATM,TO,AK,CIP,LVX,OFX	1	II	+	+	+
9	3	B	Sputum	IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	II	+	-	+
			Wounds/abscess	-	2	none	+	+	+
			Bronchial secretion	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ		II	+	+	+
10	3	B	Urine	-	2	none	+	+	-
			Urine	-		none	+	+	+
11	2	A	Urine	-	1	III	+	-	+
		B	Urine		3	II	+	+	+
12	1	A	Bronchial secretion	-	2	II	+	+	+
13	2	A	Sputum	-	2	Ia	-	+	+
		B	Urine	FEP,ATM,GM,TO,AK,CIP,LVX,OFX		none	+	-	+
		A	Bronchial secretion	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX	1	Ia	+	-	+
		A	Bronchial secretion	-	2	Ia-Ib	+	+	+
		A	Not specified	-	3	II	+	+	+
		B	Urine	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	none	+	-	+
		B	Sputum	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	Ia	+	-	+
		B	Urine	-	3	II	+	+	+
14	14	B	Urine	-	2	II	+	+	+
		B	Sputum	-	1	none	+	+	+
		B	Sputum	-	2	II	+	-	+
		B	Bronchial secretion	-	1	II	+	+	+
		B	Tracheal secretion	-	2	II	+	+	+
		B	Urine	-	1	II	+	+	+
		B	Bronchial secretion	-	1	none	+	+	+
		B	Urine	-	3	II	+	-	+
		A	Body fluid	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	Ia - III	+	-	+
		A	Sputum	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	Ia	+	+	+
15	5	A	Wounds/abscess	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	Ia	+	-	-
		A	Bronchoalveolar lavage	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX	2	Ia	+	+	+
		B	Urine	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	II	+	+	+
		A	Sputum	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	Ia	-	-	+
16	4	A	Tracheal secretion	-	2	II-Ia-Ib	+	+	+
		B	Urine	ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	III	+	+	+
		B	Urine	-	2	II	+	+	+
17	2	B	Urine	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	none	+	-	+
			Bronchial secretion	-	3	II	+	+	+
			Urine	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX	1	Ia - III	+	-	+
			Bronchial secretion	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX	1	Ia	+	-	+
18	5	B	Sputum	-	2	none	+	+	+
			Bronchial secretion	-	1	II	+	+	+
			Bronchial secretion	-	2	II	-	+	+
19	2	A	Urine	-	1	Ia-Ib	-	+	+
			Bronchial secretion		2		+	+	+
			Wounds/abscess	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	Ia - Ib	+	-	+
20	4	A	Urine	ATM,IMI,MER,CN,TO,AK,OFX,PTZ	3	II - Ia - Ib - III	+	-	+
			Wounds/abscess	-	1	II - Ia - III	+	-	-
			Urine	-	1	Ia	-	+	-
			Urine	FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ		II - Ia - Ib	+	-	+
21	3	A	Urine	FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX	1	II	+	-	+
			Urine	-		II-Ia-Ib	+	+	+
		A	Urine	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX	2	II	+	+	-
		A	Urine	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX	1	II - Ia -V	+	+	+
		A	Urine	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX	1	II	+	+	+
		A	Bronchial secretion	CAZ,FEP,ATM,IMI,MER,CN,TO,CIP,LVX,OFX,PTZ	1	II	+	-	+
22	9	A	Not specified	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	3	II	+	+	+
		A	Not specified	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX	2	III - V	+	+	+
		A	Wounds/abscess	-	2	III	+	+	+
		A	Bronchoalveolar lavage	-	3	II	+	+	+
		B	Wounds/abscess	-	3	II	+	-	-
23	1	A	Not specified	-	2	II			
		B	Wounds/abscess	IMI,MER,CN,TO,CIP,LVX,OFX	2	II	+	-	+
24	3	A	Bronchoalveolar lavage	ATM,CN,TO,CIP,LVX,OFX,PTZ	2	II	+	-	+
		A	Urine	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	II - V	+	+	+
25	2	A	Blood	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	Ia	-	-	-
			Bronchial secretion	-	2	II	+	+	+
26	2	A	Blood	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	Ia	-	-	-

26	2	A	Not specified	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	Ia	-	-	-
27	2	B	Bronchial secretion	-	2	II	+	+	+
			Sputum	-	2	II	+	+	+
28	1	B	Wounds/abscess	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	II	+	-	+
29	1	A	Catheter	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	Ia	+	-	+
30	2	A	Catheter	-	1	II-Ia	+	+	+
		B	Urine	-	4	II	+	+	+
31	1	B	Wounds/abscess	-	2	II	+	+	+
32	2	B	Wounds/abscess	CAZ,FEP,IMI,CN,CIP,LVX,OFX	2	none	+	+	+
			Bronchial secretion	-		II	+	+	+
33	1	B	Wounds/abscess	-	3	none	+	+	+
34	5	A	Bronchial secretion	-	2	II	+	+	+
		A	Bronchial secretion		1	II	+	+	+
		A	Not specified		3	II	+	+	+
		A	Not specified		2	II	+	+	+
		B	Bronchial secretion		1	none	+	+	+
35	5	A	Blood	-	1	II	-	-	-
			Sputum		1	II	-	-	+
			Urine		2	II	+	+	+
			Urine		1	II	+	-	+
			Urine		1	II	-	+	+
36	2	A	Bronchial secretion	CAZ,FEP,ATM,IMI,MER,CN,TO,CIP,LVX,OFX	1	II	+	+	-
		B	Urine	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	II	+	-	+
37	2	A	Tracheal secretion	-	1	II	+	-	+
			Urine	-		II	+	+	+
38	1	A	Urine	CAZ,FEP,ATM,CN,TO,AK,CIP,LVX,OFX,PTZ	2	II - Ia - Ib - V	+	-	+
39	3	A	Urine	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	V	+	+	+
		A	Wounds/abscess	-	2	II	+	+	-
		B	Urine	-	2	II	+	+	+
40	12	A	Body fluid	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	III	-	-	-
		A	Urine	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	III	-	-	-
		A	Urine	AK,CIP,LVX,OFX	1	Ia	-	-	+
		A	Bronchial secretion	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	II - III	-	-	-
		A	Bronchial secretion	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	Ia - III	-	-	-
		A	Catheter	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	III	+	-	-
		A	Sputum	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	II - III	+	-	+
		A	Bronchial secretion	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	III	+	+	+
		A	Bronchoalveolar lavage	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	III	+	-	+
		B	Catheter	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	III	+	-	-
		A	Sputum	-	1	II	+	-	-
		A	Bronchial secretion	-	1	III	+	+	-
41	1	A	Bronchial secretion	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	II - III	-	-	+
42	1	A	Wounds/abscess	-	2	II	+	-	+
43	1	A	Not specified	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	II - Ia - III	-	-	-
44	2	B	Wounds/abscess	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	3	II	+	+	+
			Wounds/abscess	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	none	+	-	+
45	3	B	Urine	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	III	+	-	-
			Bronchial secretion	ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	4	II	+	-	+
			Bronchial secretion	ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	II	+	+	+
46	2	A	Tracheal secretion	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	III	+	-	+
		B	Urine	FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX	3	II	+	+	+
47	2	A	Wounds/abscess	-	1	III-V	+	+	+
		B	Bronchial secretion	-	1	II	+	-	+
48	1	B	Bronchial secretion	-	2	none	+	+	+
49	2	A	Body Fluid	-	1	III	+	+	+
			Blood	-	2	Ia	-	-	+
50	1	A	Wounds/abscess	-	2	II	+	+	+
51	4	A	Wounds/abscess	FEP,ATM,IMI,MER,CN,AK,LVX,OFX,PTZ	2	II - III - V	+	+	+
			Bronchial secretion	-	1	V	+	+	+
			Body fluid	-	1	II	+	+	+
			Wounds/abscess	-	1	III	+	+	+
52	1	A	Urine	ATM,IMI,MER,CN,TO,AK,OFX,PTZ	1	II - Ia -Ib	+	+	+
53	2	A	Bronchial secretion	-	3	II	-	-	+
		B	Wounds/abscess	-	2	none	+	+	+
54	1	A	Wounds/abscess	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	Ia	+	-	+
55	1	A	Bronchoalveolar lavage	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	Ia	+	-	+
56	1	A	Urine	-	1	Ia-Ib	+	-	+
57	4	A	Wounds/abscess	-	1	II	+	+	+
			Sputum	-		none	+	+	+
			Wounds/abscess	-		II	+	-	+
		B	Bronchial secretion	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX		II - Ia	+	-	+
58	2	A	Urine	-	2	II	+	-	-
		B	Urine		2	II	+	+	+
59	4	A	Wounds/abscess	-	2	II - V	+	+	+
		A	Catheter		1	II	+	+	-
		B	Urine		1	II	+	+	+
		B	Wounds/abscess		3	none	+	+	+
60	3	A	Wounds/abscess	ATM,CN,TO,AK,CIP,LVX,OFX	3	II	+	+	+
		B	Urine	ATM,IMI,MER,TO,AK,CIP,LVX,OFX	1	Ib	+	-	+
		B	Urine	-	2	none	+	+	+
61	2	B	Bronchial secretion	FEP,ATM,CN,TO,AK,CIP,LVX,OFX,PTZ	2	Ib	+	+	+
			Bronchial secretion	-	4	none	+	+	+
62	1	A	Sputum	-	1	V	-	+	+
63	2	B	Bronchial secretion	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	none	+	+	+

63	2	B	Urine	-	2	II	+	+	+
			Bronchial secretion	CAZ,FEP,ATM,CN,TO,CIP,LVX,OFX,PTZ	2	II	+	+	+
64	3	B	Urine	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX	1	none	+	-	-
			Wounds/abscess	-	3	none	+	+	+
65	1	B	Urine	CAZ,FEP,ATM,CN,TO,CIP,LVX,OFX	2	II	+	+	+
66	1	B	Urine	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	2	none	+	+	+
67	1	B	Wounds/abscess	CAZ,FEP,ATM,CN,TO,LVX,OFX,PTZ	3	II	+	+	+
68	2	A	Tracheal secretion	-	2	II	-	-	+
			Body Fluid	-		II	+	-	-
69	1	B	Wounds/abscess	-	4	II	+	+	+
70	1	B	Blood	CAZ,FEP,IMI,MER,CN,TO,AK,CIP,LVX,OFX	3	II	+	+	+
71	6	A	Catheter	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	III	+	+	+
			Sputum		2	Ia - V	-	+	+
			Bronchial secretion		1	Ia-Ib	+	+	+
			Wounds/abscess	-	3	II	+	+	+
			Tracheal secretion		4	II	+	+	+
			Not specified		2	II	+	+	+
72	1	A	Bronchial secretion	CAZ,FEP,ATM,IMI,MER,CN,TO,AK,CIP,LVX,OFX,PTZ	1	III	-	-	+