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MICROBIOLOGY

Investigation of the association of virulence genes and biofilm production with infection and bacterial colonization processes in multidrug-resistant *Acinetobacter* spp.

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Abstract: The aim of this study was to evaluate the phenotypic and molecular patterns of biofilm formation in infection and colonization isolates of *Acinetobacter* spp. from patients who were admitted in a public hospital of Recife-PE-Brazil in 2018-2019. For the biofilm phenotypic analysis, *Acinetobacter* spp. isolates were evaluated by the crystal violet staining method; the search of virulence genes (*bap*, *ompA*, *epsA, csuE* and *bfmS)* was performed by PCR; and the ERIC-PCR was performed for molecular typing. Amongst the 38 *Acinetobacter* spp. isolates, 20 were isolated from infections and 18 from colonization. The resistance profile pointed that 86.85% (33/38) of the isolates were multidrug-resistant, being three infection isolates, and two colonization isolates resistant to polymyxin B. All the isolates were able to produce biofilm and they had at least one of the investigated virulence genes on their molecular profile, but the *bap* gene was found in 100% of them. No clones were detected by ERIC-PCR. There was no correlation between biofilm formation and the resistance profile of the bacteria, neither to the molecular profile of the virulence genes. Thus, the ability of *Acinetobacter* spp. to form biofilm is probably related to the high frequency of virulence genes.

Key words: *Acinetobacter* spp., bacterial colonization, biofilm, ERIC-PCR, hospital-acquired infection, virulence genes.

INTRODUCTION

The genus *Acinetobacter* is constituted by Gram-negative coccobacilli which are strictly anaerobic, immobile, non-fermentative, catalase positive and oxidase negative (Lee et al. 2017). Amongst the 63 validly named species, *Acinetobacter baumannii* is the most clinically relevant pathogen. Along with the species *Acinetobacter pitti*, *Acinetobacter nosocomialis, Acinetobacter calcoaceticus, Acinetobacter seifertii* and *Acinetobacter dijkshoorniae*, it constitutes the *Acinetobacter calcoaceticus-baumannii* (ACB) complex, and it is a pathogen potentially associated to healthcare-associated infections (HAI) (Nemec et al. 2015, Vijayakumar et al. 2019).

Acinetobacter spp. strains, especially *Acinetobacter baumannii,* are considered a threat to public health in a global scenario mainly due to its tendency of acquiring resistance mechanisms. This feature favors its survival even under the use of selective antimicrobial agents, and therefore, disseminates multidrug-resistant strains (Reena et al. 2017). In Brazil, it was observed that the rate of *Acinetobacter* spp.

which were resistant to carbapenems was 77.7% on adult intensive care units (ICUs), being the highest rate among Gram-negative bacilli (Brasil 2017). Besides that, *Acinetobacter* spp. has the ability of forming biofilm, which is one of the main virulence factors involved in bacterial resistance to antimicrobial agents, as it also facilitates bacterial colonization, treatment complications and persistence of these pathogens in hospitals and medical equipment (Lee et al. 2017).

The biofilm consists in a microbial community attached to the involved surface by an extracellular matrix (Flemming et al. 2016). The ability of bacterial adhesion, and the formation and development of the biofilm involve complex regulatory networks which coordinate the gene expression related to adhesion, motility and synthesis of the matrix components (Rumbo-Feal et al. 2013). Among the virulence genes related to biofilm formation in *Acinetobacter* spp., the genes *bap*, *ompA*, *epsA, csuE* and *bfmS* can be highlighted (Thummeepak et al. 2016, Zeighami et al. 2019).

Pilis production is required for biofilm formation (Amin et al. 2019). This structure is formed through the secretion system CsuA/BABCDE, regulated by the two-component system *bfmRS*, which is relevant for both formation and maintenance of *Acinetobacter* spp. biofilm on inanimate surfaces (Harding et al. 2018). The gene *csuE* encodes part of the assembling system Csu-pilis and acts as an adhesin that binds to the surface in the beginning of the biofilm formation process (Turton et al. 2007). Besides that, the protein OmpA, among other functions in bacterial pathogenesis, acts on the adhesion between the pathogen and the human epithelial cells, and the extracellular exopolysaccharide (EPS) encoded by the gene *epsA* is also involved on the cell-surface and cell-cell adhesion, protecting the bacteria against the defense mechanisms of the host. Both of them induce biofilm formation (Thummeepak et al. 2016).

The Bap protein, encoded by the *bap* gene, is a surface adhesin involved on the intracellular adhesion in mature biofilm and biomass volume. This protein is considered a key component for biofilm formation in *A. baumannii* and it might also have a role on the infection persistence caused by the bacteria (Rumbo-Feal et al. 2013).

The prevalence of colonization cases by *A. baumannii* are higher when compared to infection (Martins & Barth 2013). The ability of colonizing, combined to the multidrug resistance of this microorganism, constitute predisposing factors to the development or aggravation of infections, and also, favor the survival of the pathogen and its dissemination in hospital units (Badave & Kulkarni 2015, Ryu et al. 2017).

Therefore, the present study aimed to phenotypically characterize biofilm formation in infection and colonization isolates of *Acinetobacter* spp., and to verify the occurrence of virulence genes (*bap*, *ompA*, *epsA, csuE* and *bfmS*), describing the clonal relationships among the isolates.

MATERIALS AND METHODS

Bacterial isolates

Acinetobacter spp. isolates were acquired from patients admitted in different sectors of a public hospital in Recife, Pernambuco, Brazil. The isolates were harvested from surveillance culture samples and infection sites during the period of November, 2018 to January, 2019. The definitions of colonization and infection followed the clinical and microbiological criteria of the hospital. The clinical isolates were sent to the Bacteriology and Molecular Biology Laboratory of the Medical Science Center/UFPE, where they were conserved in glycerol and stored at -80 °C.

The bacteria were reactivated after incubation in test tubes containing brain-heart infusion (BHI) broth at 35 ± 2 °C for 48 hours. Then, they were seeded in Luria Bertani (LB) medium and incubated at 35 ± 2 °C for 24 hours. Regarding bacterial identification, *Acinetobacter* spp. isolates were identified by the automated system BD Phoenix® prior to acquisition, and afterwards, submitted to a molecular identification through the detection of the $bla_{oxa\text{-}51\text{-}like}$ gene to confirm the species *A. baumannii*, according to Woodford et al. (2006).

Susceptibility profile to antimicrobial agents

The susceptibility analysis of the isolates was performed through the automated system BD Phoenix®, which tested the antimicrobial agents amikacin, cefepime, ceftazidime, ceftriaxone, ciprofloxacin,gentamicin,imipenem,levofloxacin, meropenem, piperacillin/tazobactam and trimethoprim + sulfamethoxazole. Afterwards, the minimum inhibitory concentration (MIC) of polymyxin B to *Acinetobacter* spp. isolates was determined through the broth microdilution assay, according to the Clinical and Laboratory Standards Institute (CLSI 2019). *Escherichia coli* NCTC 13846 was used as the positive control and *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used as negative controls. All the experiments were performed in triplicate in three independent days.

The isolates were considered multidrug resistant (MDR), multidrug sensitive (MDS) and the extensively drug resistant (XDR) according to Magiorakos et al. (2012).

Phenotypic characterization of biofilm formation

To evaluate biofilm formation, the crystal violet staining method was used according to Stepanović et al. (2000). *Acinetobacter* spp. isolates were cultured in Tryptic Soy Broth (TSB) at 35 ± 2 °C for 24 hours. The microtitration was performed in triplicate in three independent days using 96-well flat-bottom polystyrene plates, where 200 µL of the bacterial suspensions at 0.5 on the McFarland scale were added, using the TSB broth without inoculum as the negative control. As the positive control, the strain *A. baumannii* ATCC 19606 was used. The plates were then incubated at 35 ± 2 °C for 24 hours. After this period, the bacterial suspensions were removed, and each well was washed 3 times with 250 µL of a sterile 0.9% NaCl solution. Then, the fixation was performed with 200 µL of 99% methanol for 15 minutes. After that, the methanol was removed, the plates were dried at room temperature and further stained with 200 µL of 1% crystal violet solution for 20 minutes. The plates were then washed with running water and dried at room temperature. After this process, the plates were read in an ELISA plate reader (BioRad, model 550) at 570 nm wavelength. The values of the optical densities of each isolate (ODi) were obtained by the mean of the triplicates, comparing this value with the optical density of the negative control (ODc). The isolates were classified in four categories according to the mean optical densities (OD) related to the results obtained for the ODc. The categories were based on the following criteria: non adherent if ODi ≤ ODc; weakly adherent (+) if ODc < ODi ≤ 2 x ODc; moderately adherent (++) if 2 x ODc < ODi ≤ 4 x ODc; or strongly adherent (+++) if 4 x ODc < ODi.

Total DNA extraction

After incubating the isolates in Luria Bertani (LB) broth at 35 ± 2 °C for 24 hours, the total DNA extraction was performed using the Brazol kit (LGC-Biotechnology), according to the manufacturer's instructions. The extracted DNA was quantified (NanoDrop 2000c, Thermoscientific®) and stored at -20 °C.

Detection of virulence genes

The presence of the virulence genes *bap*, *ompA*, *epsA, csuE* and *bfmS* was detected through polymerase chain reaction (PCR) with the *primers* and amplification conditions described on Table I. Each reaction was prepared in a final volume of 25 µL per tube, which comprised of 20 ng (2 µL) of the total DNA, 15 pmol (1 µL) of each *primer*, 0.6 µL of deoxyribonucleotide triphosphate (dNTP) (8 mM), 5.0 mL of buffer (5×), 2.0 mL of $MgCl_2$ (25 mM) and 0.2 µL of *Taq* DNA Polimerase (5U). The PCR products were submitted to electrophoresis in a 1% agarose gel with 0.5× TBE buffer, and after staining with *blue-green* (LGC Biotecnology, São Paulo, Brazil) , they were visualized and photodocumented in an ultra-violet transilluminator (Kasvi®, Brazil).

Table I. List of primers and amplification conditions for the search of virulence genes in *Acinetobacter* spp.

Gene	Oligonucleotide sequence (5´-3´)	Amplification condition	Amplicon (bp)	Reference
Bap	**F:** TACTTCCAATCCAATGCTAGGGAGGGTACCAATGCAG **R:** TTATCCACTTCCAATGATCAGCAACCAAACCGCTAC	Initial denaturation 96 °C, 5 min, 35x: 96 °C, 1 min; 56.5 °C, 1 min; 72 °C, 1 min, Final extension: 72 °C, 10 min	1225	Goh et al. (2013)
csuE	**F:** ATGCATGTTCTCTGGACTGATGTTGAC **R:** CGACTTGTACCGTGACCGTATCTTGATAAG	Initial denaturation 96 °C, 5 min, 35x: 96 °C, 1 min; 57 °C, 1 min; 72 °C, 1 min, Final extension: 72 °C, 10 min	976	Turton et al. (2007)
ompA	**F:** CGCTTCTGCTGGTGCTGAAT **R:** CGTGCAGTAGCGTTAGGGTA	Initial denaturation 94 °C, 5 min, 35x: 94 °C, 1 min; 55 °C, 1 min; 72 °C, 45 sec, Final extension: 72 °C, 5 min	531	Tayabali et al. (2012)
bfmS	**F:** TTGCTCGAACTTCCAATTTATTATAC **R:** TTATGCAGGTGCTTTTTTATTGGTC	Initial denaturation 94 °C, 5 min, 35x: 94 °C, 1 min; 55 °C, 1 min; 72 °C, 45 sec, Final extension: 72 °C, 5 min	1368	Liou et al. (2014)
epsA	**F:** AGCAAGTGGTTATCCAATCG **R:** ACCAGACTCACCCATTACAT	Initial denaturation 94 °C, 5 min, 35x: 94 °C, 1 min; 55 °C, 1 min; 72 °C, 45 sec, Final extension: 72 °C, 5 min	451	Tayabali et al. (2012)

F: forward, R: reverse, bp: base pairs.

A. baumannii ATCC 19606 was used as a positive control.

Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR)

The search for the genetic relationship between the isolates was performed through ERIC-PCR. The reactions were prepared in a total volume of 25 µL per tube, corresponding of: 100 ng of the genomic DNA, 10 pmol of the primers (ERIC-1 [5'- ATGTAAGCTCCTGGGGATTCAC-3']; ERIC-2 [5'-AAGTAAGTGACTGGGGTG AGCG-3']), Buffer (1x), 200 µM of deoxyribonucleotide triphosphate, 1.5 mM of $MgCl_2$ and 1U of *Taq* DNA polymerase. The amplification parameters used for the ERIC-PCR were: initial denaturation at 95 ºC for 3 minutes, followed by 30 cycles of denaturation at 92 °C for 1 minute, annealing at 36 °C for 1 minute and extension at 72 °C for 8 minutes. After the 30 cycles, a final extension of 16 minutes at 72 °C was performed. The PCR products were stained with blue-green (LGC Biotecnology, São Paulo, Brazil) and submitted to electrophoresis in a 1.5% agarose gel, visualized and photodocumented under UV light (Kasvi®, Brazil) (Duan et al. 2009). The data analysis and dendrogram constructions were performed using the software Past. The similarity adopted to define the clonal relationship amongst the *Acinetobacter* spp. strains was equal or higher than 85%.

Statistical analysis

The statistical analyzes were conducted using the software Graphpad prism 8.3.1. The sample description was performed by frequency with a 95% confidence interval ($CI_{95\%}$). The difference of the biofilm's biomass among infection and colonization isolates was compared using the Student's t-test. The Chi-squared test was used to analyze the differences between the frequencies of virulence genes related to biofilm formation. P values <0.05 were considered statistically significant.

Ethics approval

This study was approved by the Ethics Committee of the Health Sciences Center - Federal University of Pernambuco, under the number: CAAE 0490.0.172.000-11.

RESULTS

A total of 38 isolates of *Acinetobacter* spp. from patients admitted in a public hospital in Recife-PE, Brazil, were collected between 2018 and 2019. Among them, 20 were infection isolates, being 45% (9/20) from tracheal secretion, 30% (6/20) from blood, 20% (4/20) from catheter tips and 5% (1/20) from urine. The remaining 18 isolates were collected from surveillance cultures, being 94.44% (17/18) from rectal swab and 5.56% (1/18) from nasal swab, characterized as colonization isolates. The species *Acinetobacter baumannii* was identified in 81.58% (31/38) of the isolates in this study by investigating the presence of the $bla_{\text{OXA-51-like}}$ gene. The seven strains which were negative for the $bla_{\text{OXA-51-like}}$ gene were identified as being from the *Acinetobacter calcoaceticus-baumannii* (ACB) complex, according to the analysis on BD Phoenix.

The antimicrobial susceptibility profiles of *Acinetobacter* spp. isolates are described on Figure 1. All 38 isolates were resistant to third-generation cephalosporins (ceftazidime and ceftriaxone), piperacillin/tazobactam, fluoroquinolones (ciprofloxacin and levofloxacin) and carbapenems (imipenem and meropenem). Based on the resistance profile of the isolates evaluated in this study, 86.85% (33/38) were characterized as MDR. Both infection and colonization isolates had a resistance rate to amikacin and cefepime equal or higher than

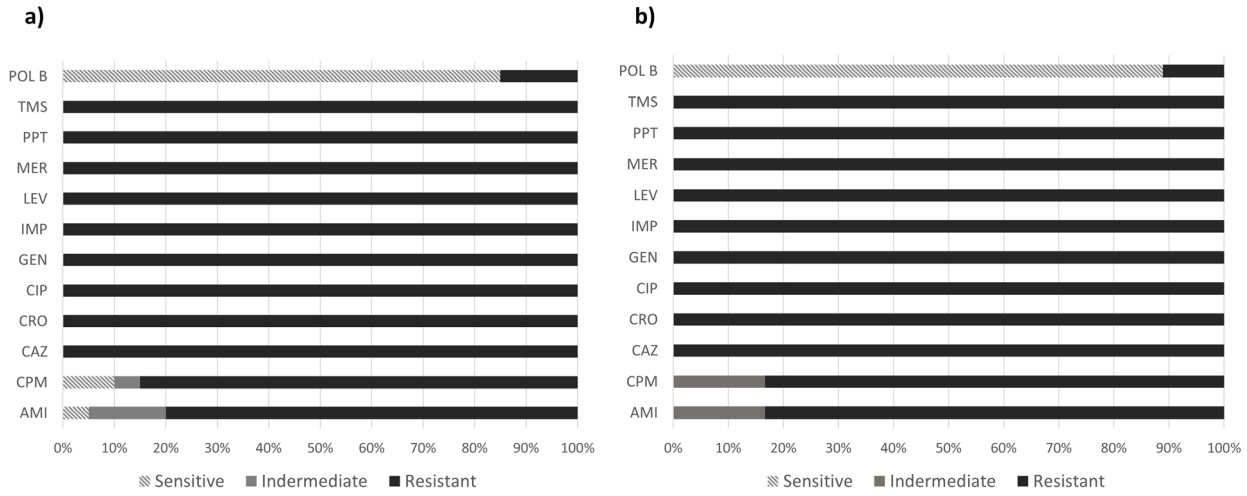

Figure 1. Percentage representation of antimicrobial susceptibility profiles of *Acinetobacter* spp. isolates in Recife – PE – Brazil. a) Infection isolates and b) Colonization isolates. AMI: amikacin; GEN: gentamycin; IMP: imipenem; MER: meropenem; CIP: ciprofloxacin; LEV: levofloxacin; CPM: cefepime; CAZ: ceftazidime; CRO: ceftriaxone; PPT: piperacillin/tazobactam; TRI/SUL: trimethoprim + sulfamethoxazole; POL B: polymyxin B.

80%. Besides that, the MIC values of polymyxin B determined by the broth microdilution assay varied from 0.125 to 32 µg/mL, being 85% (17/20) and 88.88% (16/18) classified as sensitive isolates from infection and colonization, respectively. Therefore, although most clinical *Acinetobacter* spp. isolates presented growth inhibition against polymyxin B at concentrations of 0.25 to 1 µg/mL, 15% (3/20) of the infection isolates and 11.11% (2/18) of the colonization isolates were resistant to polymyxin B, being classified as XDR.

Through the evaluation of the phenotypic profiles of *Acinetobacter* spp. biofilms, it was demonstrated that all 38 isolates had different adherence intensities. The comparison between biofilm formation among infection and colonization isolates of *Acinetobacter* spp. according to the intensity categories of adherence, did not present statistical significance by the Student's t-test ($p = 0.378$; $CI_{95\%}$ -0.015 – 0.039), as it can be observed on Figure 2. Amongst the 20 infection isolates, 5 (25%) were characterized as weakly adherent (3 MDR and 2 XDR), 11 (55%) as moderately adherent (11 MDR) and 4 (20%) as strongly adherent (3 MDR and 1 XDR). Regarding the 18 colonization isolates, 6 (33.33%) were classified as weakly adherent (5 MDR and 1 XDR), 10 (55.56%) as moderately adherent (9 MDR and 1 XDR), and 2 (11.11%) as strongly adherent (2 MDR).

No statistical difference was observed when the resistance profile of the microorganism was correlated with biofilm formation according to the Chi-squared test ($p > 0.05$). Infection and colonization isolates presented similar percentages of weak, moderate and strong biofilm phenotypic profiles, as well as the XDR profile (Figure 3).

The amplification of the virulence genes (*bap*, *ompA*, *csuE*, *bfmS* and *epsA*) by PCR pointed that all the 38 *Acinetobacter* spp. clinical isolates carried at least one of these genes related to biofilm formation. The most prevalent gene was *bap,* which was present in all 38 *Acinetobacter* spp. isolates. The occurrence of the genes *ompA, csuE*, *bfmS* and *epsA* among the infection isolates was of 95% (19/20), 85% (17/20), 70% (14/20) and 65% (13/20), respectively. Among the colonization isolates, the frequency of the virulence genes was of 94.44% (17/18)

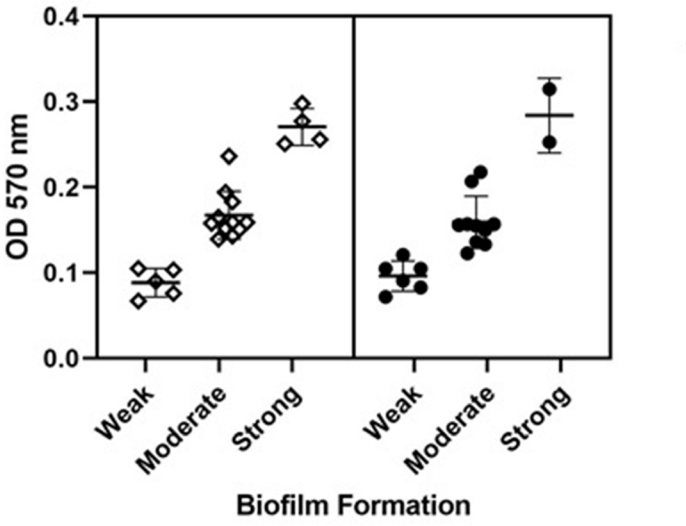

Figure 2. Comparison of the phenotypic profiles of *Acinetobacter* spp. biofilms, distributed as infection and colonization isolates in Recife– PE – Brazil.

for *ompA* and *csuE*, followed by 61.11% (11/18) for *bfmS* and *epsA*. From all the 5 virulence genes which were studied, in 85% (17/20) of the infection isolates and in 94.44% (17/18) of the colonization isolates, the genes *bap*, *ompA* and *csuE* coexisted in their genetic profile.

Likewise, there was no statistical significance between infection and colonization samples when the frequency of the virulence genes (*bap*, *ompA*, *epsA, csuE* and *bfmS*) and the biofilm phenotypic profile were evaluated by the Chi-squared test ($p > 0.05$) (Figure 4).

In the present study, the genotyping of clinical *Acinetobacter* spp. isolates by ERIC-PCR identified 38 distinct genetic profiles, with similarities ranging from 20% to 75% (Figure 5).

DISCUSSION

The incidence of HAIs caused by species of the genus *Acinetobacter* is increasing on the global scenario, and in spite of accounting for over 50 validly named species, *Acinetobacter baumannii* is the predominant pathogen isolated from patients in hospital environments (Li et al. 2016, Wong et al. 2017). In this study, through the phenotypic and molecular analysis of the $bla_{\text{OXA-51-like}}$ gene, 81.58% (31/38) of the isolates were identified as *A. baumannii* and 18.42% (7/38) as part of the *Acinetobacter calcoaceticus-baumannii* complex (ACB). In 2019, Falah et al. (2019) have reported that 97.56% (80/82) of *A. baumannii* isolates identified by phenotypic and biochemical tests, carried the $bla_{\text{OXA-51}}$ gene, confirming therefore, the identification of the isolates as *A. baumannii*.

The antimicrobial susceptibility profile revealed that 86.85% (33/38) of *Acinetobacter* spp. isolates were MDR. All the 38 isolates were resistant to third-generation cephalosporins, fluoroquinolones and carbapenems, which are considered the main therapy for infections caused by *A. baumannii* (Amin et al. 2019, Silva & Domingues 2017). Both infection and colonization isolates presented a resistance rate to amikacin and to cefepime equal or higher than 80%. Similar results were previously reported in studies performed in several countries, including Brazil (Badave & Kulkarni 2015, Duarte et al. 2016, Fallah et al. 2017, Lima et al. 2020).

In accordance with the high rates of resistance to carbapenems found in this study, Wasfi et al. (2021) detected resistance to carbapenems meropenem and ertapenem in 70.8% (34/48)

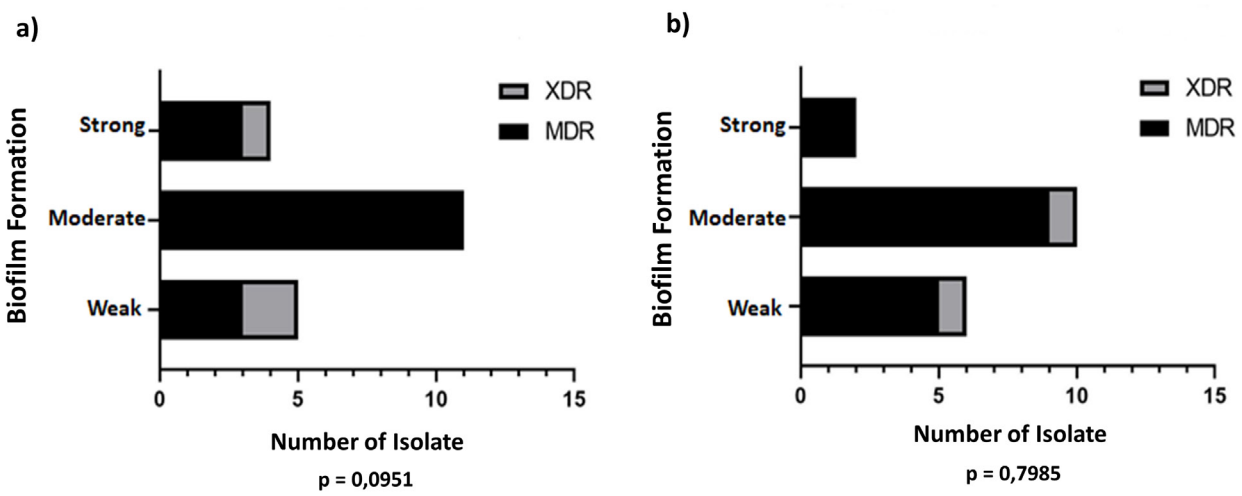

Figure 3. Correlation between biofilm formation and resistance profiles of *Acinetobacter* spp. isolates in Recife – PE – Brazil. a) Infection isolates and b) Colonization isolates.

of *A. baumannii* isolates collected from blood infection samples, in cancer patients, at the National Cancer Institute (NCI), Giza, Egypt. This resistant profile presented by Gram-negative bacilli such as *Acinetobacter* spp. concerns the global public health because if there is a high isolation of strains which are resistant to carbapenems, the treatment options get more limited and will induce, therefore, the need of new therapeutic approaches, such as the use of polymyxins (colistin and polymyxin B) (Duarte et al. 2016). In this study, although more than 85% of the isolates were sensitive to polymyxin B, 15% (3/20) of the infection isolates and 11.11% (2/18) of the colonization isolates were resistant, and thus, classified as XDR. There is a variation on the incidence or frequency of polymyxin B resistance in *Acinetobacter* spp. isolates in Brazil. Romanin et al. (2019) pointed that 10.7% (11/103) of *A. baumannii* isolates harvested from blood samples of patients admitted in a hospital in Londrina - Brazil, were resistant to polymyxin B. On the other hand, Siqueira et al. (2018) identified that all 68 *A. baumannii* samples obtained from urine, blood and other secretions, isolated in a hospital from Espírito Santo - Brazil, were susceptible to polymyxin B.

The phenotypic analysis has indicated that all 38 *Acinetobacter* spp. isolates in this study were biofilm producers with similar percentages of weak, moderate and strongly adherent among infection and colonization isolates. Zeighami et al. (2019) have demonstrated that all *A. baumannii* isolates obtained from blood, thorax and urine secretions were capable of producing biofilm, however, 58% (58/100) of these isolates were strongly adherent, whereas in the present study, the same was verified in only 20% (5/20) of infection isolates and in 11.11% (2/18) of colonization isolates. Besides that, there was no difference on the OD means of the biofilms when it was compared between isolates grouped according to the source (infection and colonization). Similarly, when biofilm formation was investigated in clinical and environmental isolates of *A. baumannii* in Iran, Ghasemi et al. (2018) also did not detect differences based on the sample, although environmental isolates present a higher ability of producing biofilm compared to clinical ones, what reinforces the colonization potential of this bacteria.

According to Schroeder et al. (2017), virulence is not only defined by the ability of colonization, but also for causing diseases,

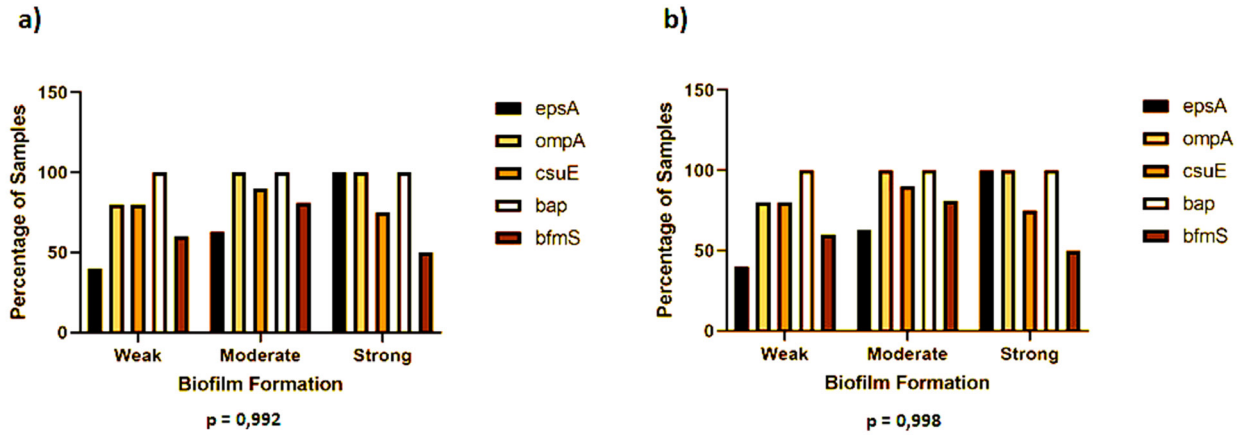

Figure 4. Comparison between biofilm formation and frequency of virulence genes in *Acinetobacter* spp. isolates in Recife – PE – Brazil. a) Infection isolates and b) Colonization isolates.

and the biofilm formation is an essential part of bacterial pathogenesis with evidences that its development provides a higher resistance to antimicrobial agents. While some authors have found a positive relationship between the MDR profile of *A. baumannii* isolates and biofilm formation (Badave & Kulkarni 2015, Duarte et al. 2016, Yang et al. 2018), others have identified the opposite, in which an increased biofilm formation is higher among non-MDR isolates of *A. baumannii* (Aminm et al. 2019, Qi et al. 2016). Although there are divergent reports about the relationship between the resistance profile and biofilm formation, this study did not observe a significant relationship between these characteristics among *Acinetobacter* spp. isolates, corroborating with Lima et al. (2020), whose analysis of 35 strains of *Acinetobacter baumannii* harvested from tracheal secretion, urine and cerebrospinal fluid has revealed that 24 isolates (68.6%) were strong biofilm producers (23 XDR and one non-MDR); 10 isolates (28.6%) were moderate producers (9 XDR and one non-MDR); and only one isolate (2.9%) was a weak biofilm producer with an XDR profile.

Among the virulence genes involved in biofilm formation, the *bap* gene was the most prevalent in this experimental study, being present in all 38 isolates of *Acinetobacter* spp. The high prevalence of the *bap* gene was also reported by Goh et al. (2013) and Liu et al. (2016), who have detected the *bap* gene in 91.7% (22/24) and 95.5% (84/88) of *A. baumannii* strains, respectively, and have shown the importance of the Biofilm associated protein (Bap), expressed on the cell surface, contributing for the biofilm formation *in vitro*.

In the present study, the genes *csuE, ompA, epsA* and *bfmS* presented high frequency rates among *Acinetobacter* spp. isolates. Whilst the prevalence of the genes *ompA* and *csuE* ranged from 85% to 95%, the prevalence of *bfmS* and *epsA* genes ranged from 61.11% to 70%, with similar frequencies among infection and colonization isolates. The prevalent gene in a study performed by Zeigmani et al. (2019) was *csuE*, present in all 100 isolates of *A. baumannii*, corroborating with the high percentages found in our results. In 2016, Thummeepak et al. (2016) have described the distribution of the virulence genes *ompA, bfmS* and *epsA* in *A. baumannii* isolates with frequencies of 84.4% (190/225), 84% (189/225) and 22.2% (50/225), respectively, and revealed a high prevalence of biofilm producing profiles among these isolates.

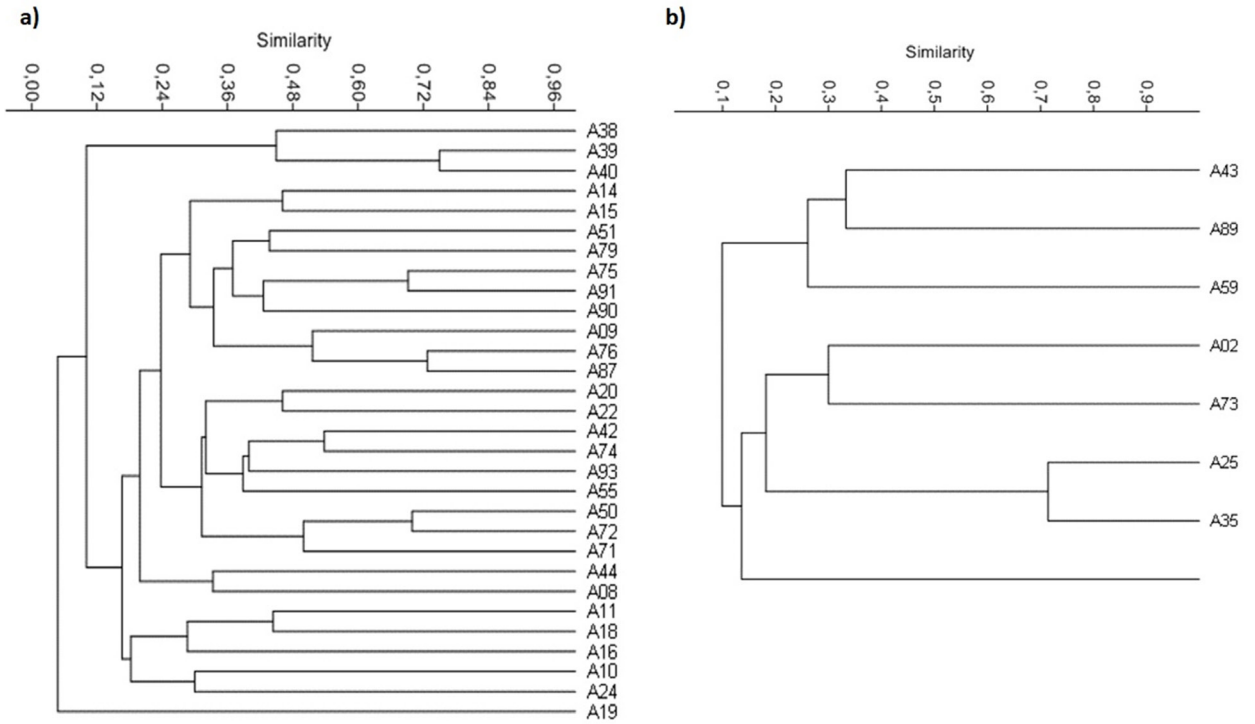

Figure 5. Dendrogram representing the patterns of Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) from *Acinetobacter* spp. clinical isolates in Recife – PE – Brazil. a) *Acinetobacter baumannii* isolates and b) *Acinetobacter calcoaceticus-baumannii* complex isolates.

When analyzing the correlation between biofilm formation and the related virulence genes, Yang et al. (2018) have suggested that *A. baumannii* strains which carry the genes *bap, ompA* and *csuE* tend to produce strongly adherent biofilms when compared to isolates without these genes. Although these genes have coexisted in 85% (17/20) of the infection isolates and in 94.44% (17/18) of the colonization isolates, this study did not find a significant correlation between biofilm formation and the presence of the virulence genes, neither a statistical difference when infection and colonization isolates were compared.

According to the condition of the biofilm formation, the genetic products expressed by the genes are crucial for adhesion and biofilm development, being a determinant factor on the epidemiology of infections caused by *Acinetobacter* spp. (Siqueira et al. 2018). Liou et al. (2014) have demonstrated that, for instance, the *bfmS* mutated gene decreased biofilm formation, reduced the adherence to cells and the sensitivity to serum killing of *A. baumannii*. In this case, the previous identification of virulence genes in colonization samples contaminated by *Acinetobacter* spp. can indicate the risk of infection in patients who are colonized with virulent isolates able to produce biofilm, since in most outbreaks of *A. baumannii*, colonized patients are in general, a source of initial transmission (Falah et al. 2019).

The phylogenetic relation among the isolates was investigated by ERIC-PCR. This molecular typing method was performed for being fast, reliable and of easy execution for the epidemiological elucidation of strains that circulate in hospital environments (Amin et al. 2019). According to the results, this method indicated genetic diversity and heterogeneity

among the 38 clinical *Acinetobacter* spp. isolates, with a similarity ranging from 20% to 75%. However, no clone was identified. Previous studies have reported the identification of clonal profiles in *Acinetobacter baumannii* isolates, as Moreira et al. (2018) who detected 17 different groups among 48 isolates; Falah et al. (2019) who identified 14 ERIC-PCR patterns among 80 isolates; and Heidari et al. (2018) who identified 20 ERIC-PCR types among 75 isolates. Paradoxically, although there are reports of clonality in the literature, it is also possible to recognize the level of clonal diversity among the strains of *A. baumannii*.

CONCLUSIONS

In Brazil, there is a lack of reports regarding the analysis of biofilm formation and the detection of virulence genes in *Acinetobacter* spp. strains. The present study has demonstrated multidrug-resistant *Acinetobacter* spp. isolates, including those resistant to polymyxin B. It was also evidenced the high phenotypic prevalence of biofilm formation, high frequencies of virulence genes and the heterogeneity between infection and colonization isolates. Therefore, the use of surveillance cultures for the continuous monitoring of the epidemiological and susceptibility profiles to antimicrobials used against circulating microorganisms in hospital environments, could be one of many strategies to guide the therapy of nosocomial infections caused by bacteria and minimize the propagation of virulent strains.

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AMCMS designed the study, executed the laboratorial methodology, analyzed the data and wrote the manuscript. SDCJ, JLCL and JLBFF have contributed by performing the phenotypical characterization of biofilm formation, PCR and antimicrobial assays. MAVM and IMFC have supervised the laboratory work and contributed with the critical evaluation of the manuscript. All authors have read and approved the article's submission.