



ANTIMICROBIAL RESISTANCE AND FORMATION OF BIOFILM IN CLINICS ISOLATES CAUSERS OF INFECTIONS RELATED TO HEALTH CARE

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ABSTRACT

Healthcare-related infections (HAI) represent a great risk to their victims and are an increasing cost to the clinical and therapeutic setting. The fast increase in microbial resistance to antibiotics in the hospital environment decreases drug effectiveness, increases length of stay, and increases the cost of treatment. Besides that, it may result in premature death as a direct consequence of unsafe health practices, according to the World Health Organization. In this context, the present study aimed to evaluate microbial resistance and biofilm formation in isolates of clinical interest. The methodology used was based on the analysis and identification of clinical isolates of *Enterococcus faecalis*, *Enterococcus faecium*, *Proteus mirabilis* and *Providencia stuartii*, performed by the Vitek® 2 Compact automated system (bioMérieux) and confirmed by obtaining mass spectrum in MALDI-TOF apparatus. Autoflex III Mass Spectrometer which were compared with MALDI Biotyper version 3.1 database. Microbial resistance was evaluated by Minimum Inhibitory Concentration (MIC) and biofilm formation of these isolates by violet crystal method, Congo Red Agar and optical microscopy. As results, it was observed that all isolates tested were compatible with identification by MALDI-TOF. In addition, of all isolates tested, 83.3% had higher resistance to the antibiotic Ampicillin (AMP) and 58.3% showed resistance to Ciprofloxacin (CIP), ranging from Resistant Multidrug (MDR) to Extensively Drug Resistant (XDR), by various other antibiotics. All microorganisms presented biofilm formation ranging from moderate to strong, in which the strong ones represented 66.6% of isolates. It was observed that the identification of pertinent characteristics to microorganism might provide an effective therapy focused in reducing the infectious diseases related to HAI.

Keywords: Bacteria. MALDI-TOF. Therapy.

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INTRODUCTION

Infectious diseases caused by bacteria still represent an important cause of morbidity and mortality among humans worldwide, what makes antimicrobial resistance become a public health problem¹. From this perspective, the control of infections caused by pathogens multidrug resistant represents a challenge for public health services worldwide². According to data from the World Health Organization (WHO), in countries with high income of money, one in 10 hospitalized patients is affected by infections related to health care (HAIS), while developing countries suffer with higher HAI load, which can be up to 20 times higher than in developed countries³. Epidemiological studies of this magnitude reveal the urgent increase in need for therapeutic alternatives that acts as effective agents in combating infections, suggested by Resolution - ANVISA in Brazil⁴. In the environment inside a hospital, there is a greater likelihood of being infected by a healthcare-associated infection (HAI), caused by the growing bacterial resistance mechanisms against exogenous factors and reduced quantity of antibiotics that are efficient against these pathogens⁵. The means to contain the risks associated with these injuries, such as correct hygiene, contribute to reducing the frequency of HAIs by an additional 50%¹. On that context, the use of tools that allow the elucidation and knowledge about alternatives to combat HAI becomes a necessary and constant battle, in order to understand the intrinsic resistance and the adaptation mechanisms that the microorganisms are taking for increasing resistance to antimicrobials⁶. In the hospital environment, resistant microorganisms are capable of presenting multiple virulence mechanisms and pathogenic versatility in opportunistic character⁶.

The use of implantable biomaterials is a constant problem at HAI, since which weakens natural protection barriers and facilitates the appearance of infections and development of bacterial biofilms⁷. Among the mechanisms of

bacterial aggregation, biofilms are responsible for increasing the acquired resistance of microorganisms and exchanging genes that provide resistance to antibiotics used in the clinic. Due to these characteristics, infections associated with biomaterials have come to be recognized as one of the biggest clinical problems⁸.

Therefore, the objective of this work was to evaluate the resistance profile and the formation biofilm of clinical isolates from hospitals in the metropolitan region of Recife city, PE, where the high incidence of health-related infections (HAI).

MATERIAL AND METHODS

Biological material and cultivation conditions

Clinical isolates of *Enterococcus faecalis*, *Enterococcus faecium*, *Proteus mirabilis* and *Providencia stuartii* were collected from hospitals in the metropolitan area of Recife, PE. The isolates were maintained in 15 % glycerol (-80 °C) and reactivated in Brain Heart Infusion (BHI) at 37 °C / 24h. The Informed Consent Form (ICF) was not necessary, as the isolates were obtained from routine diagnostics and data processed anonymously. The process for obtaining isolates was approved by the Research Ethics Committee, under the document number 2.581.723/2.581.568.

MALDI-TOF Mass Spectrometry

Bacterial colonies were resuspended in 300 µL of Milli-Q water, and then, 900 µL of absolute ethanol was added. Suspensions were centrifuged at 15,600 turns for 2 min, the supernatant removed and the pellet was dried in SpeedVac for 20 min. Then, 50 µL of 70 % formic acid, 50 µL of acetonitrile were added to homogenized mixture on a vortex mixer, centrifuged at 15,600 g for 2 min and the supernatant transferred to a new microtube. The matrix was prepared with alpha-cyano-4-hydroxycinnamic acid (10 mg / mL) in 50 % acetonitrile and 0.3 % trifluoroacetic acid, and applied to the MALDI plate with the sample at room temperature to crystallization. The

acquisition of MS spectra in positive linear mode (Voltage of acceleration: 20 kV and detection range - m/z : 2,000 - 20,000) was performed by the Program Flex Control Version 3.0 in MALDI-TOF Autoflex III Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). The obtained mass spectra were compared with the MALDI Biotyper Version 3.1 Database.

Susceptibility profile

Isolates were tested for susceptibility to different antimicrobials: Amikacin (AMI), Ampicillin (AMP), Benzylpenicillin (PEN), Cefepime (CPM), Cefoxitin (FOX), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefuroxime (CFX), Ciprofloxacin (CIP), Daptomycin (DTA), Erythromycin (ETA), Ertapenem (ETP), Streptomycin (ETT), Gentamicin (GEN), Imipenem (IPM), Linezolid (LIL), Meropenem (MEM), Moxifloxacin (MFA), Norfloxacin (NFA), Piperacillin / Tazobactam (PTZ), Teicoplanin (TPA), Tigecycline (TGA), Tobramycin (TBA) and Vancomycin (VCA). Experiments were carried out in triplicate using antibiogram, according to the Clinical and Laboratory Standards Institute - CLSI⁹.

Congo red agar test

The evaluation of polysaccharides production to constitute the biofilm matrix was performed using the Congo red agar method, following the protocol described by Freeman¹⁰. Congo red dye was used as a pH indicator, black in pH ranges between 3.0 and 5.2. Agar plates Congo red, were sown and incubated 24 hours at 37 °C. After this period, colonies of dark red or blackish color, with dry or crystalline consistency, were considered to be biofilm producers, while red colonies with smooth and darkened in the center were considered not to produce biofilm. It was used the reference strain ATCC *Pseudomonas aeruginosa* (PA01) as a positive control of test, as it is characterized as a biofilm producer.

Biofilm formation

The formation of biofilm by the isolates was verified in microtiter plates, by the violet crystal method described by Stepanovic¹¹. In BHI, the

mean values of absorbance of each sample (OD a) compared to the absorbance of the control of sterility (OD c). All strains were classified into the following categories: non-adherent (0), adherent weak (+), moderate (++) or strongly (+++), based on the OD's of bacterial films. We defined the control OD (OD c) for the microtiter as three standard deviations above the mean OD of the negative control. At strains were classified as follows: $OD \leq ODC$ non-adherent $ODC < OD \leq 2 ODC$ weakly adherent $2 \times ODC < OD \leq 4 \times ODC$ moderately adherent $4 \times ODC < OD$ strongly adherent OD.

Optical Microscopy

For visualization of the biofilm formation, isolates that were the biggest biofilm builders in the previous tests were chosen, strains (A) *Proteus mirabilis* strain 11720. (B) *Providencia stuartii* strain 499.4. (C) *Enterococcus faecalis* strain 890.6. (D) *Enterococcus faecium* cepa 17.6. Three coverslips were placed in each petri dish, which contained half BHI. In the control of Sterility 10 ml BHI medium was added. While, on the test plates, 9 mL of culture medium and 1 ml of the bacterial inoculum were added. The plates were incubated at 37 °C for 24 hours, and after this process, the contents were removed and the coverslips washed three times with 0.85 % saline. For fixing the biofilm the coverslips were incubated at 55 °C for 1 h. Later, they were stained with 0.4 % violet crystal for 15 min, and subsequently washed three times with water to visualization of the biofilm in an inverted microscope, the coverslips were placed on slides and dried for 5 minutes at 55 °C, using a 40x magnification for visualization.

Statistical analysis

The experimental design was completely randomized, with three replications, for verification of biological effects. Analysis of Variance (ANOVA) was applied to verify effects of treatments, the averages were compared using the Tukey test, establishing a level of significance of 95 % or $p < 0.05$. Simple linear regression was used (SPSS 15.0 software; BioEstat 4.0) to find the inhibitory concentration

for 50 % of inhibition. The graphs and IC 50 values and their respective 95 % confidence limits (CL 95 %) were elaborated using the PrismGraphPad 6 software.

RESULTS AND DISCUSSION

The MALDI-TOF MS, in the clinical context, presents itself as an innovative technology for the fast and accurate identification of microorganisms. Thus, this technique contributes to reducing the time of mortality and hospitalization of patients and, consequently,

has a relevant impact on public health¹². The strains from different hospitals in the Metropolitan Region of Recife were obtained from different sectors and sites of infection. Our results were completed by 66.6 % of strains that came from urine culture, 16.6 % from surgical wounds, 8.4 % from catheter tips, and 8.4 % from peritoneal liquid, as can be seen in Table 1. According to Magiorakos et al., (2012)¹³, the isolates were characterized as multidrug resistant (MDR), extensively drug-resistant (XDR) and Pan drug-resistant (PDR).

Table 1: Characteristics related to the resistance profile and biofilm formation of the isolates from hospitals of Recife, PE.

Isolated	Compatibility	Hospital Sector	Infection Site	Antimicrobial Classification	Biofilm Formation	Red Congo Ágar
24.717	<i>E. faecalis</i>	VASC	Peritoneal liquid	MDR	Strong	Negative
98.717	<i>E. faecalis</i>	UTI-A	Urine	MDR	Weak	Positive
890.617	<i>E. faecalis</i>	CM	Urine	MDR	Strong	Positive
17.617	<i>E. faecium</i>	EC	Urine	MDR	Moderate	Positive
543.417	<i>E. faecium</i>	ORT	Urine	MDR	NF	Positive
882.617	<i>E. faecium</i>	EC	Urine	MDR	Weak	Positive
392.4	<i>P. mirabilis</i>	UTI-A	Urine	XDR	Strong	Positive
11720	<i>P. mirabilis</i>	CM	Cateter	XDR	Strong	Positive
14047	<i>P. mirabilis</i>	EG	Wound	MDR	Strong	Positive
198.4	<i>P. stuartii</i>	ORT	Wound	MDR	Strong	Positive
426.4	<i>P. stuartii</i>	UTI-P	Wound	XDR	Strong	Positive
499.4	<i>P. stuartii</i>	UTI-P	Wound	XDR	Strong	Positive

VASC = vascular; ICU-A = adult intensive care unit; CM = Medical Clinic; EC = Cardiological Emergency; ORT = Orthopedics; EG = General Emergency; ICU-P = Pediatric intensive care unit; NF = Non-former; MDR = Resistant Multidrug; XDR = Extensively Drug Resistant.

In this study, Table 2, the bacteria of the genus *Enterococcus* spp (*E. faecalis* and *E. faecium*), showed compatibility MDR, whereas for those of the Enterobacteriaceae Gram negative family (*P. mirabilis* and *P. stuartii*), there was a predominance of species XDR. These data corroborate the growing profile of resistance that bacteria have been acquiring, making it increasingly necessary to establish guidelines aimed at reducing this problem.

In addition, according to Table 1, the hospital sectors with the highest incidence of

XDR microorganisms were the adult and pediatric ICUs, which are aggravating factors for the establishment of an effective therapy, since the patient is already in a situation of clinical weakness. Resistant microorganisms represent a challenge for the control of HAIs in hospitals, mainly in ICUs¹⁴. The main resistant bacteria are represented by CESP group enterobacteria (*Citrobacter* spp, *Enterobacter* spp, *Serratia* spp, *Proteus* spp, *Providencia* spp and *Enterococcus*¹⁴).

Table 2: Profile of susceptibility of isolates from hospitals of Recife, PE to several antibiotics.

Isolado	Identificação	AMI	AMP	CPM	FOX	CRO	CFX	CIP	ETA	ETP	ETT	GEN	IPM	LIL	MEM	MFA	PTZ	TPA	TGA	TBA	VCA
24.717	<i>E. faecalis</i>	-	S	-	-	-	-	R	R	-	R	S	-	S	-	S	-	S	S	R	S
98.717	<i>E. faecalis</i>	-	R	-	-	-	-	R	R	-	S	R	-	S	-	R	-	S	S	R	S
890.617	<i>E. faecalis</i>	-	S	-	-	-	-	R	R	-	R	R	-	S	-	R	-	S	S	R	S
17.617	<i>E. faecium</i>	-	R	-	-	-	-	R	R	-	R	S	-	S	-	R	-	S	S	R	S
543.417	<i>E. faecium</i>	-	R	-	-	-	-	R	R	-	R	-	-	S	-	R	-	S	S	R	S
882.617	<i>E. faecium</i>	-	R	-	-	-	-	R	R	-	R	S	-	S	-	R	-	S	S	R	S
392.4	<i>P. mirabilis</i>	S	R	I	S	R	R	R	-	R	-	I	R	-	R	-	-	-	-	-	-
11720	<i>P. mirabilis</i>	I	R	S	S	I	R	I	-	R	-	R	R	-	R	-	R	-	-	-	-
14047	<i>P. mirabilis</i>	S	R	R	S	S	R	S	-	-	-	R	-	-	S	-	S	-	-	-	-
198.4	<i>P. stuartii</i>	S	R	S	S	S	S	S	-	-	-	R	-	-	S	-	S	-	-	-	-
426.4	<i>P. stuartii</i>	S	R	S	S	I	R	S	-	-	-	R	I	-	S	-	S	-	-	-	-
499.4	<i>P. stuartii</i>	S	R	I	S	I	R	S	-	-	-	R	R	-	S	-	S	-	-	-	-

Amikacin (AMI), Ampicillin (AMP), Benzylpenicillin (PEN), Cefepime (CPM), Cefoxitin (FOX), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefuroxime (CFX), Ciprofloxacin (CIP), Daptomycin (DTA), Erythromycin (ETA), Ertapenem (ETP), Streptomycin (ETT), Gentamicin (GEN), Imipenem (IPM), Linezolid (LIL), Meropenem (MEM), Moxifloxacin (MFA), Norfloxacin (NFA), Piperacillin / Tazobactam (PTZ), Teicoplanin (TPA), Tigecycline (TGA), Tobramycin (TBA) and Vancomycin (VCA). R= Resistant; S= Susceptible; I= Indifferent.

Table 2 shows the greater effectiveness of antibiotics Teicoplanin, Tigecycline and Vancomycin tested for Gram positive. Gram negative microorganisms demonstrate greater susceptibility to Cefoxitin. Due to the fact that the collection of strains occurs in different hospitals in the metropolitan region of Recife, there was no total standardization regarding the use of antibiotics. The qualitative test (Figure 1) of Congo red was described by Freeman (1989) and is easy to perform, fast, sensitive and reproducible, presenting as one of the additional advantages the fact that the colonies remain viable in the environment. The positive reaction,

evidenced by the darkening of the biofilm producing colony, due to the polysaccharide constitution of the extracellular matrix of the biofilm, whose production is intensified by the nutritional supplement of the medium¹⁰ showed the isolated biofilm producers. However, there were divergences between the results obtained by this methodology and the formation of the biofilm by the violet crystal method, with the isolate 24,717 not showing the production of the polysaccharide matrix in the Congo red agar test, while in the verification proposed by Stepanovic et al (2007)¹¹ the presence of the biofilm was verified by optical density (OD).

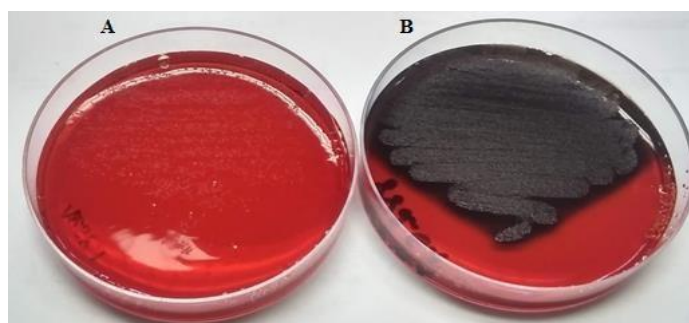


Figure 1. Visualization of biofilm formation from isolates from hospitals of Recife, PE in the Congo Red Agar Test. A = Negative result regarding the formation of Biofilm; B = Positive result regarding the formation of Biofilm.

The biofilm formation profile in polystyrene plates demonstrated a greater production by Gram negative microorganisms (*P. mirabilis* and *P. stuartii*), where they were classified as strong producers. Whereas Gram positive isolates (*E. faecalis* and *E. faecium*), showed a low production, as shown in Figure 2. However,

isolate 890.6 showed a higher production of the polysaccharide matrix. This training by clinical isolates corroborates to Macedo (2000)¹⁵, who classifies biofilms as being a concern for the pharmaceutical industry, as they allow greater resistance to the antibiotics used in the clinic.

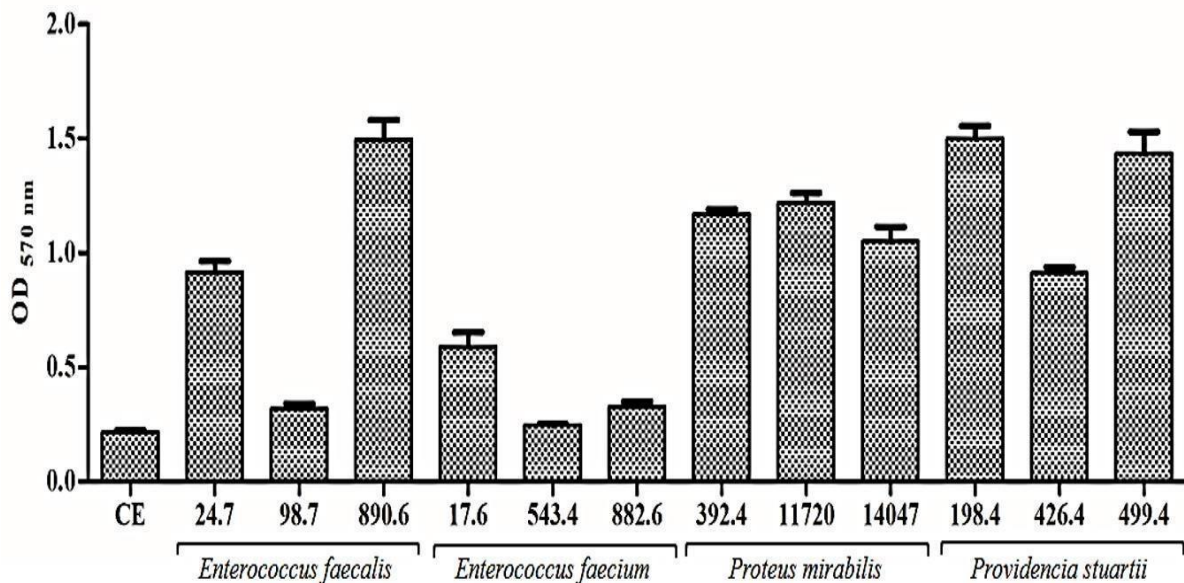


Figure 2. Biofilm formation of isolates from hospitals of Recife, PE. CE= Control of Sterility, OD= Optical Density.

In this context, isolates from catheters include *Enterococcus faecalis*, *P. mirabilis* and other species. These microorganisms may come from the skin of the patient or the multidisciplinary health team, from drinking water in which the connectors are exposed¹⁷. Figure 3 demonstrates the formation of bacterial biofilm revealed with violet crystal and visualized under reverse microscopy. In the same figure, is shown some traces of the polysaccharide matrix of the biofilm in the different isolates, having in common the intense formation of this matrix by the method described by Stepanovic et al (2007)¹¹. The isolates were observed at 40x magnification. The isolates that showed higher biofilm formation in the other methodologies were chosen for this technique, in order to better visualize the formation of the matrix. The visualization of polymeric aggregates

corroborates with Trentin et al., (2013)¹⁶, who demonstrates that these aggregates are responsible for the exchange of genes that confer resistance to microorganisms.

Biofilm formation of the microorganisms that proved to be the largest producers, stained by 0.4 % violet crystal, visualized in an inverted microscope (40x magnification). (A) *Proteus mirabilis* strain 11720. (B) *Providencia stuartii* strain 499.4. (C) *Enterococcus faecalis* strain 890.6. (D) *Enterococcus faecium* cepa 17.6.

The results obtained provided a better understanding of the profile of microbial infections, demonstrating the complexity of the microorganisms that cause these infections, as well as their resistance mechanisms by different methods. The association of methodologies allowed for a systematization of these mechanisms and their impact on public health.

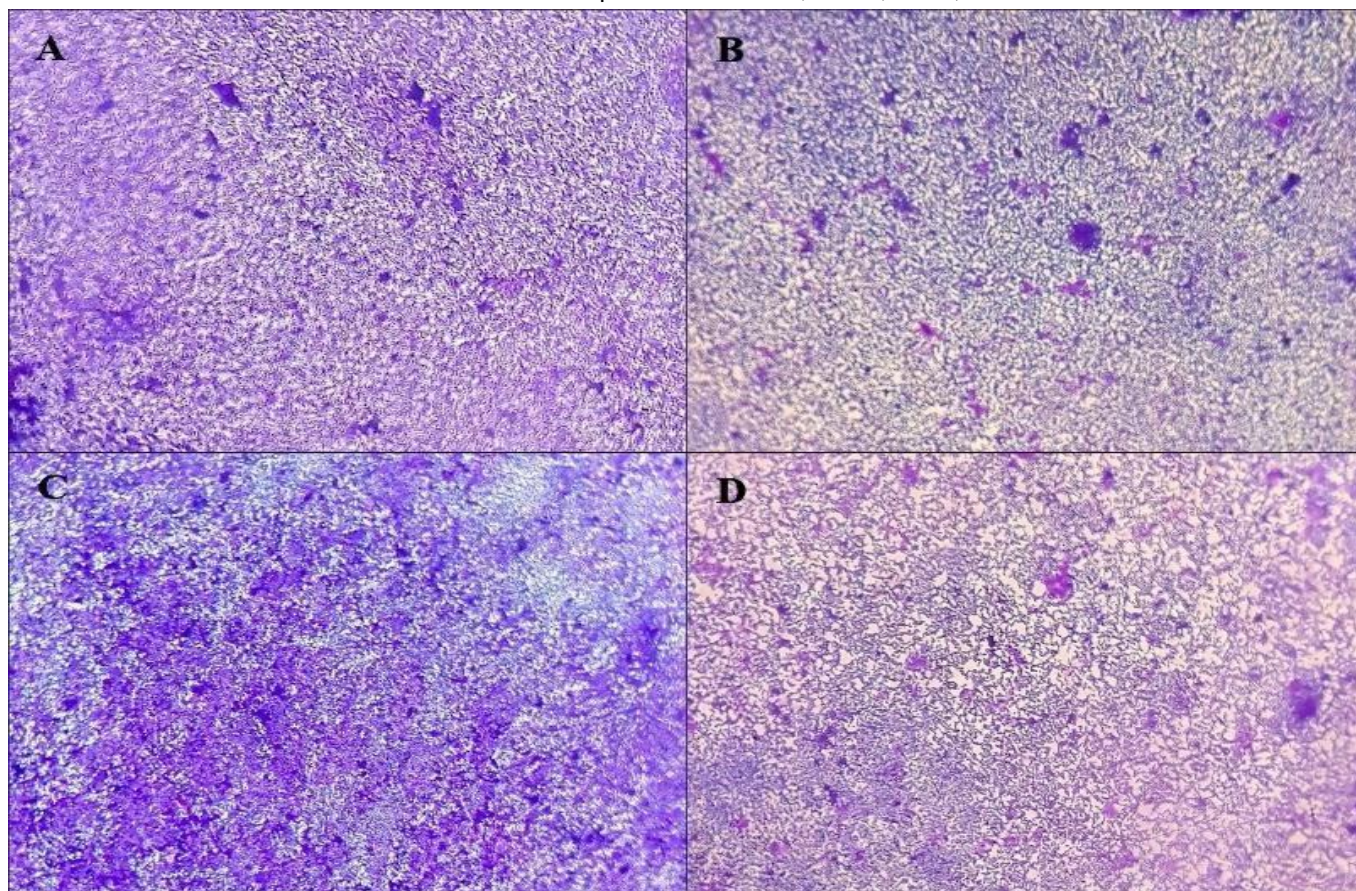


Figure 3. Microscopy of greater bacteria from hospitals of Recife, PE with biofilm capability in a coverslip with violet crystal.

CONCLUSION

Gram negative isolates showed a higher biofilm formation capacity than Gram positive isolates. Associated with this, the susceptibility profiles of all isolates showed compatibility for MDR and XDR, which are aggravating factors, and which can compromise the therapy for patients. The different methodologies for assessing the characteristics relevant to the isolates, corroborate the warning from the World Health Organization about the threat of infections related to health care, which the infection sites demonstrated by this study, may have an impact on the drug treatment that will be chosen, since the presence of these resistant isolates and biofilm builders in intensive care units, especially in pediatric units, can compromise the treatment of the children.

Moreover, resistance to the antimicrobials used in the clinic contributes to an inefficient and biased therapy to the use of a more costly

clinical approach to the patient and the governmental health system, increasing the resistance mechanisms and allowing the continuity of the cycle in which the HAIs are inserted. Therefore, an adequate therapeutic approach is necessary, using mechanisms of microbiological identification that allow the appropriate identification for a better treatment to the patient.

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