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Contribution of biofilm and Cell Surface Hydrophobicity in the resistance of *Pseudomonas aeruginosa* from some Healthcare settings of Yaounde

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ABSTRACT

Antibiotic resistance (ABR) is one of the priority problems around the world which affects the evolution of infectious diseases and leads to economic losses with a considerable impact on increased mortality and morbidity in low-resource countries. One of the bacteria most involved in resistance is *Pseudomonas aeruginosa*. It is a bacteria belonging to the ESKAPE group, for which there is an urgent and critical need for antibiotic development. The circulation of this bacteria in several countries has been the cause of many infections affecting all ages, patients and healthcare staff, and has promoted the spread of resistance in environmental and animal health. To investigate the determinants of resistance in *Pseudomonsa aeruginosa*, the aim of this work was to assess the correlation between biofilm, Cell Surface Hydrophobicity (CSH) and antibiotic resistance in clinical isolates of *Pseudomonas aeruginosa* circulating in Yaounde.

Methods: We conducted a cross sectional study from March 2019 to October 2021. We collected various samples including pus, urine, lochia, wound, bedsore and blood, from patients of the General Hospital of Yaounde (GHY), Central Hospital of Yaounde (CHY), University Teaching Hospital of Yaounde (UTHY) and Centre Pasteur du Cameroun (CPC). The hydrophobicity of the bacterial surfaces was determined by measuring the percentage of adhesion to xylene. Biofilm formation was assessed using the TCP method. The correlation between CSH, biofilm production and ABR were determined.

Results: We included 300 patients in this study. The most represented patients were men (50.67%). *P. aeruginosa* was isolated from the four settings and the prevalence was 10%. A large proportion of *P.aeruginosa* were biofilm producer with 30% being strong biofilm producer, 53.33% were weak biofilm producer and 16.67% were non-biofilm producers. Twenty percent of *P. aeruginosa* were highly hydrophobes while, 23.33% were moderately hydrophobes and 56.67 were low hydrophobes. The activities of most antibiotics were negatively correlated with biofilms and hydrophobicity. Isolates from lochia and wounds were biofilm producers and showed multidrug resistance while isolates from urine and pus was very hydrophobic.

Conclusion: These findings should serve as evidence base that biofilms and hydrophobicity are factors that contribute to antibiotic resistance in *Pseudomonas aeruginosa*. This demonstrates the need to consider these virulence factors, as well as the nature of the sample or excipient, during patient treatment, especially during empirical antibiotic therapy.

KEYWORD: Biofilm, hydrophobicity, Antibiotic resistance

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INTRODUCTION

Antibiotic resistance is one of the priority problems around the world which affects the evolution of infectious diseases by increasing virulence. This problem is present in all countries with an increased risk of clinical damage and mortality in patients. A variety of resistance-determinants have been described in previous studies, which allows bacteria to withstand lethal doses of antibiotics [1].

Pseudomonas aeruginosa is a bacteria which is part of the "ESKAPE", a list of bacteria for which there is a critical need for the development of new antibiotics [2]. This bacteria is considered as one of the priority bacteria to be monitored. It belongs to the six leading pathogens for deaths associated with resistance [3]. It is a bacteria with a level 2 epidemiological importance, level 4 cost and level 3 ease of resistance spread [4].

Most of the ESKAPE-members are biofilm-producers, and hydrophobic [1][5]. Biofilms production is known to be an important factor leading to treatment failures [1]. There have been significant interest in assessing the possible relationship between antibiotic resistance and CSH or biofilm-production in ESKAPE-members. The aim of this study is to assess the correlation between CSH or Biofilms production and antibiotic resistance in clinical isolates of *Pseudomonas aeruginosa* from some reference settings of Cameroon.

METHODS

Study design

An analytical cross-sectional study was conducted between May 2019 to October 2022 on people requesting a bacteriological analysis in the Bacteriology laboratory of GHY, CHY, UTHY and CPC. We included in this study all the presumptive infected participants who gave their written consent or assent to participate in the study and who requested a bacteriological analysis. We did not included in this study people who had not given their consent, assent or persons who did not request for bacteriological analysis. Bacteria which did not belong to the species *P. aeruginosa* were excluded. The selection of the four health care settings was carried out by a convenient sampling among the reference health facilities. The selection of patients was done by a ramdom sampling at the sampling unit of the GHY, CHY, UTHY and CPC Demographic data were collected using a questionnaire which was reviewed by the researchers themselves as well as laboratory work experts.

We determined the minimal sample size by the formula described by Cochran [6], using a prevalence of *Pseudomonas aeruginosa* infections of 23.1% [7].

Isolation

We collected pus, urine, lochia, wound, bedsore and blood samples from participants. Each sample was inoculated on Mac Conkey agar. The medium was then incubated for 24 hours at 37°C. After culture, identification of isolates, was performed by microscopy, oxidase test and Api 20 NE.

Oxidase test

The oxidase test was carried out according to the recommendation of Sagar *et al*. [8]. Briefly, a sterile filter paper was placed on a slide and then soaked with N,N,N,N-Tetramethyl-p-phenylenediamine Dihydrochloride solution. A 24-hour colony was then crushed on this filter paper. A positive reaction was characterized by the appearance of a blue colour. The absence of staining characterized a negative reaction.

Api 20 NE test

Api 20 NE was carried out according to the recommendation of the manufacturer [9]. The test consisted of 20 microtubes containing dehydrated substrates which were inoculated with a saline bacterial suspension and incubated for 24H . After incubation, metabolism produces color that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the reading table and the identification is obtained by referring to the analytical profile index or using the identification software.

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were conducted according to the recommendations of the Antibiogram committee of the French Microbiology Society using the disc diffusion method on Mueller-Hinton agar [10]. The inoculum was prepared from a pure 24-hour culture of the bacteria obtained on Mac Conkey agar. The equivalent of 4 colonies were suspended in a sterile physiological water. Bacterial suspension was adjusted to 0.5 McFarland and inoculated on Mueller-Hinton agar. Fifteen minutes later, antibiotic discs were deposited on the medium. This was left at room temperature for 15 minutes and then incubated at 37°C for 24 hours. Inhibition zones were measured after incubation and scored as susceptible, intermediate or resistant.

Cell Surface Hydrophobicity test

Hydrophobicity was measured using the Microbial Adhesion to Hydrocarbon (MATH) protocol [11], with slight modifications. A 24 H suspension of *Pseudomonas aeruginosa* standardized to 0.5 Mac Farland were prepared and the cells were harvested by centrifugation. The cell pellet was washed twice with sterile distilled water and suspended in 3mL of sodium nitrate ($NaNO_3$) and the absorbance was read at 580 nm (A_0). Then 1 mL of xylene was added to the 3 mL of cell suspension. After a 10 min of preincubation at room temperature, the two phase system was mixed by vortexing for 2 min. The two phases were allowed to separate for 30 min at room temperature; the aqueous phase was then taken carefully and absorbance was determined (A_1). Hydrophobicity index was calculated according to the formula: Hydrophobibity index (%)= $[(A_0-A_1)/A_0]x100$.

Biofilm formation test

The study of biofilm formation was carried out by the tissue culture plate method according to the recommendation O'Toole and Kolter [12]. To perform this, 150 µL of brain heart infusion broth was added to each well of a sterile microtitre plate. Then 10 µL of inoculum adjusted to 0.5 McFarland was inoculated into each well and 10 µL of autoclaved distilled water was added in the peripheral wells containing only the medium as negative control. The whole was incubated for 24 hours at 37°C. After incubation, each well was emptied gently using a syringe, avoiding contact with the wall of the well. The wells were then washed 3 times with distilled water and then stained with 1% crystal violet for 5 minutes. Then the wells were washed twice and then dried. Ethanol-acetone (75:25) was introduced into each well. The micro-well plates were agitated for one minute and the optical density was read at 570 nm. The tests were repeated three times. The categorization of isolates was done according to the recommendations of Christensen *et al*., which states: $OD \leq OD_c$: Nonbiofilm former; $OD_c < OD \leq 2 \times OD_c$: Weak biofilm-former; $OD > \times 2OD_c$: Strong biofilm-former [13]. With: $OD_c = OD_{avg} + 3$ S.D of OD_{avg}; OD: Optical density specimen; OD_{avg}: Average Optical Density of a clean tissue culture plate; OD_c: Optical density cut-off.

Statistical analysis

The data base was conceived with excel 2010. Statistical analysis was performed with graph pad prism 10.0.2. We conducted multivariate analysis using principal component analysis. Observation charts allowed us to determine the link between resistance and biofilms or hydrophobicity.

RESULTS

Samples were collected from 300 patients. The distribution of patients according to age range (Table I), showed that the most represented patients were young adults (64%), followed by older adults (21.33%). The least represented patients were infants (3.33%) followed by adolescents (3.33%). The majority of participants came from the CHY (54.33%) and the UTHY (30.66%).

Table I: Distribution of patients according to the gender, health care settings and age ranges

	Number	Frequency (%)
Gender		
Male	152	50,67
Female	148	49.33
Age range		
<1	10	3.33
[1- 12]	24	8
[13-17]	10	3.33
[18-64]	192	64
[65-100]	64	21.33
Health care settings		
Centre Pasteur du Cameroun	13	4.33
University Teaching Hospital of Yaounde	92	30.66
Central Hospital of Yaounde	163	54.33
General Hospital of Yaounde	32	10.66

The distribution of samples in each ward revealed the most frequent infections in these wards (Figure 1). Pus was the sample represented in most units, with distribution in dental (100%), gastroenterology (100%), ear, nose and throat (100%), surgery (72%), gynecology (64.28%), diabetology (33.33%), medicine (30.15%), emergency (17.24%) and intensive care wards (14.63%). Urine ranks second in terms of distribution across wards, with a representation in urology (100%), traumatology (100%), neonatology (66.67%), intensive care unit (46.34%), medicine (46.03%), emergency (41.37%), pediatric (24.13%) and surgery (16%).

The intensive care and medicine units had the highest diversity of specimens, with urine in first place in intensive care unit (46.34%) and medicine (46.03%). Pus was second (30.15%) and blood third (17.46%) in medicine. Blood and pus occupied the second (24.39%) and third (14.63%) positions respectively in intensive care unit. Outpatients presented a wide range of specimens, with 30.38% for urine, 32.91% for pus, 16.45% for nasopharyngeal, 7.59% for stool, 6.33% for vaginal secretion, 1.27% for ear swab, 1.27% for urethral fluid and 1.27% for blood.

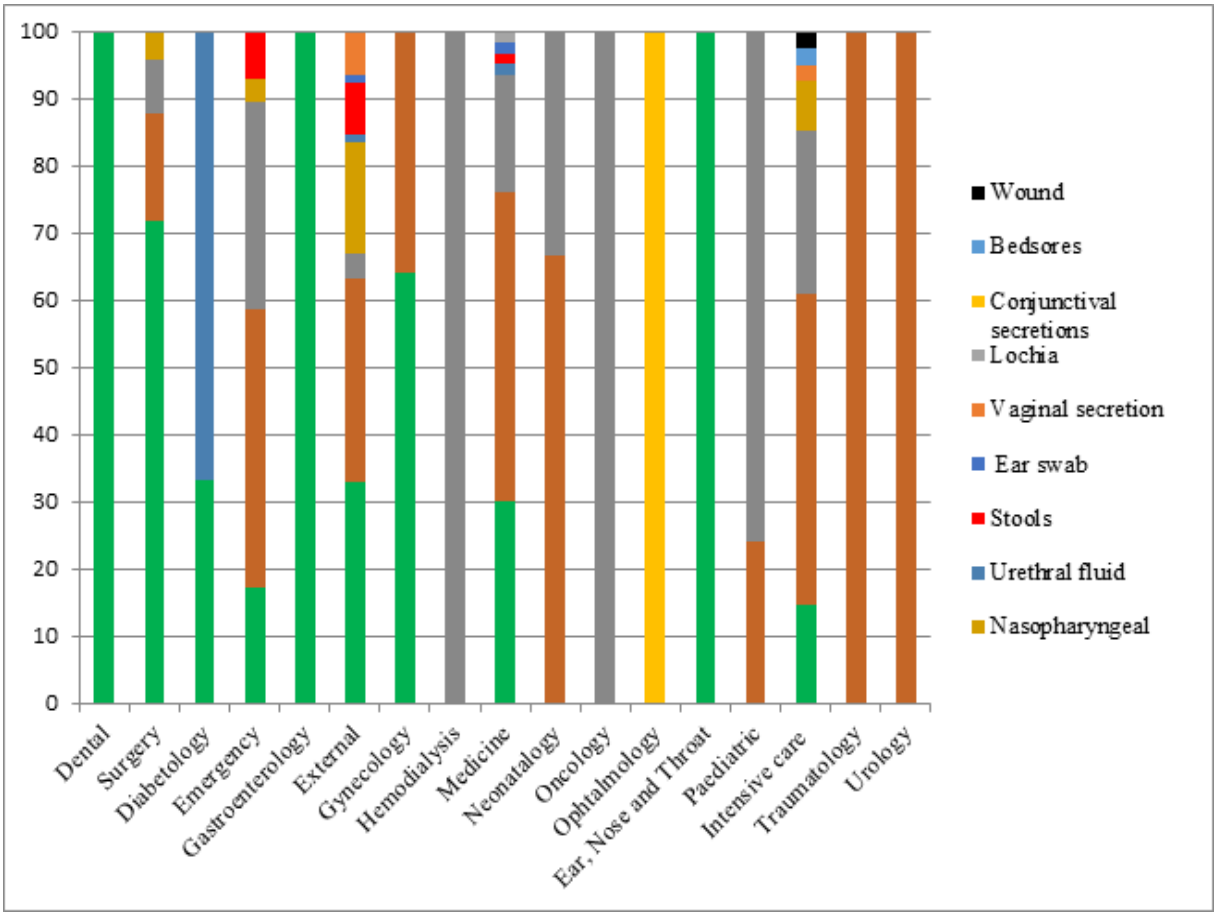

Figure 1: Distribution of samples according to the wards

Cell surface hydrophobicity of *P. aeruginosa* was determined by measuring the percentage of adhesion to Xylene (Figure 2). *P. aeruginosa* isolates showed several levels of hydrophobicity. Of the 30 isolates, 20% were highly hydrophobers, 23.33% were moderate hydrophobers and 56.67 were low hydrophobers.

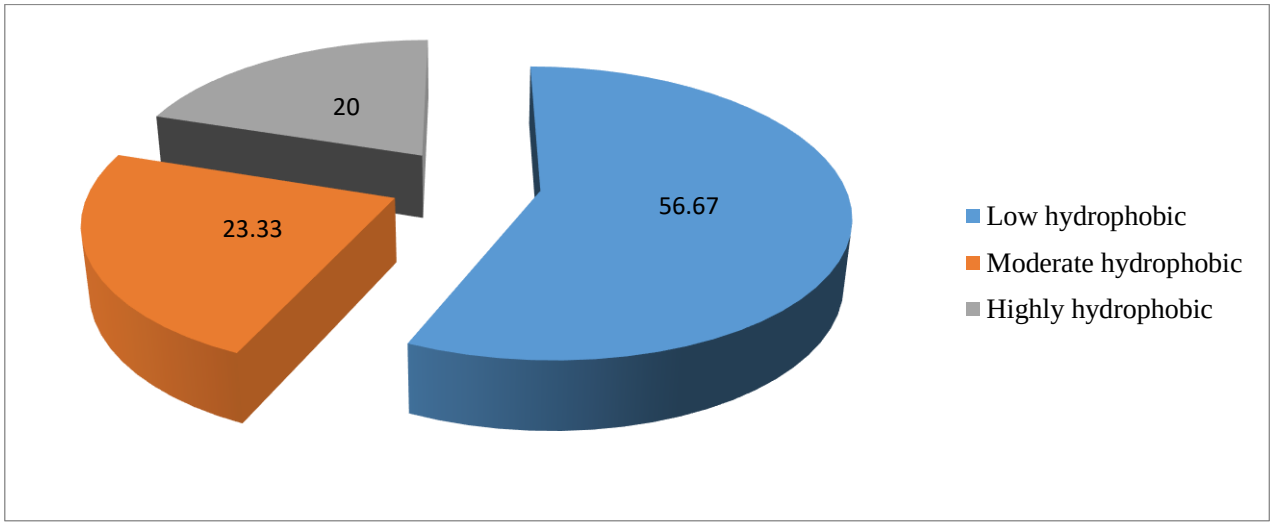

Figure 2: Frequency of P. aeruginosa according to the levels of hydrophobicity

Biofilm test was used to identify strong biofilm producers, weak biofilm producers and non-biofilm producers (Figure 3). In this study 30% of *P. aeruginosa* were strong biofilm producers, 53.33% were weak biofilm producers and 16.67% of isolates were non-biofilm producers.

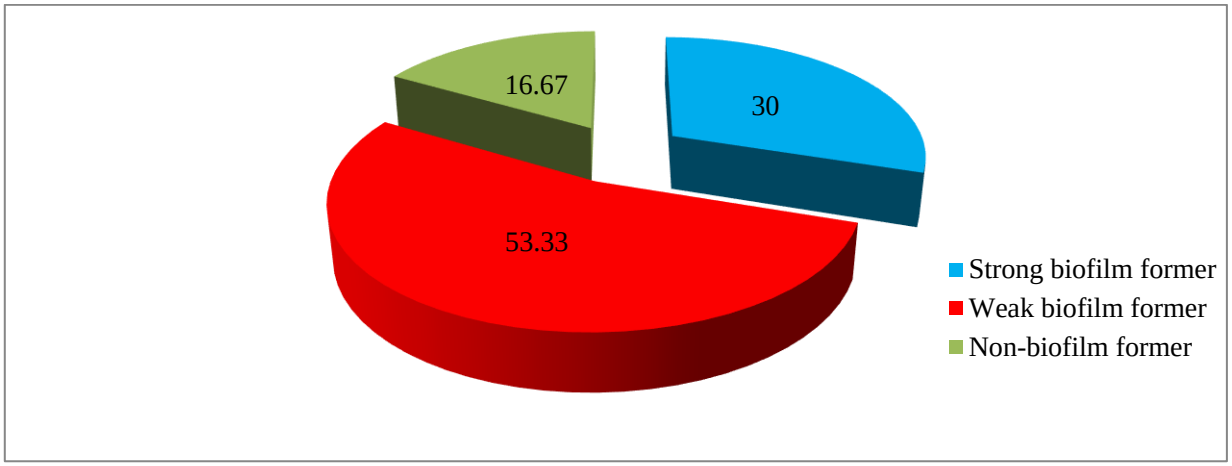

Figure 3: Frequency of biofilm production by Pseudomonas aeruginosa

Principal component analysis was used to group antibiotics with similar activity according to health facility (Figure 4). The activity of Tircacillin increases with the activity of Piperacillin/ Tazobactam, the activity of Netilmicin increases with the activity of Ciprofloxacin and Ceftazidim, and the activity of Meropenem increases with the activity of Norfloxacin and Tircacillin/Clavulanic acid. Better activities were observed in UTHY isolates with Tircacillin and Piperacillin/Tazobactam, unlike GHY where activity was better with Imipenem and CHY where activities were better with Netilmicin, Ciprofloxacin and Ceftazidim. Furthermore, the patients who came to the Centre Pasteur du Cameroun were infected with isolates resistant to most antibiotics.

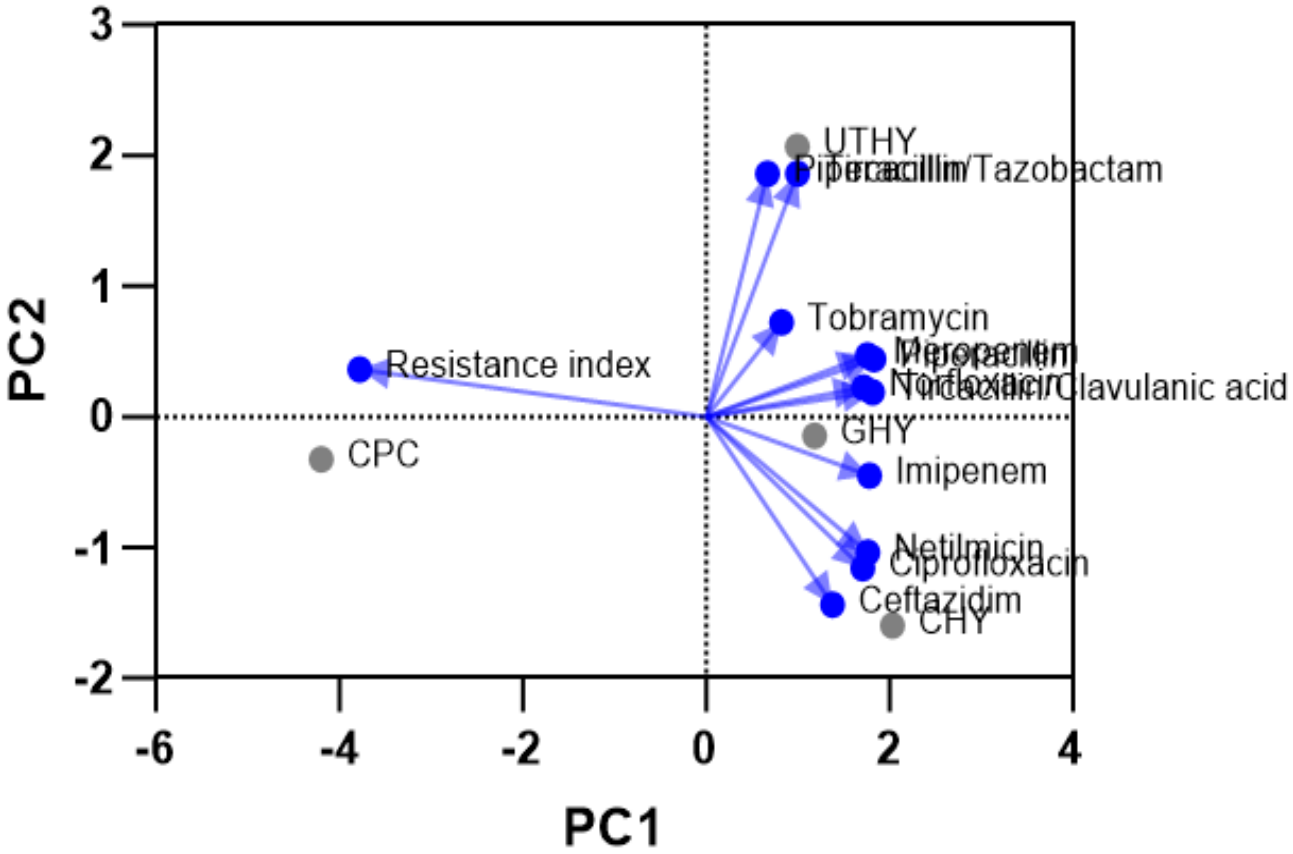

Figure 4: Correlation between antibiotic activities in settings

The correlation between antibiotic activity, hydrophobicity and biofilm production showed that isolates from adults were high biofilm-producers and only Tobramycin have a good activity in these patients (Figure 5). The activities of most antibiotics (inhibition zones) were negatively correlated with biofilm formation and hydrophobicity, with the exception of Netimicin and Imipenem, which were positively correlated with hydrophobicity.

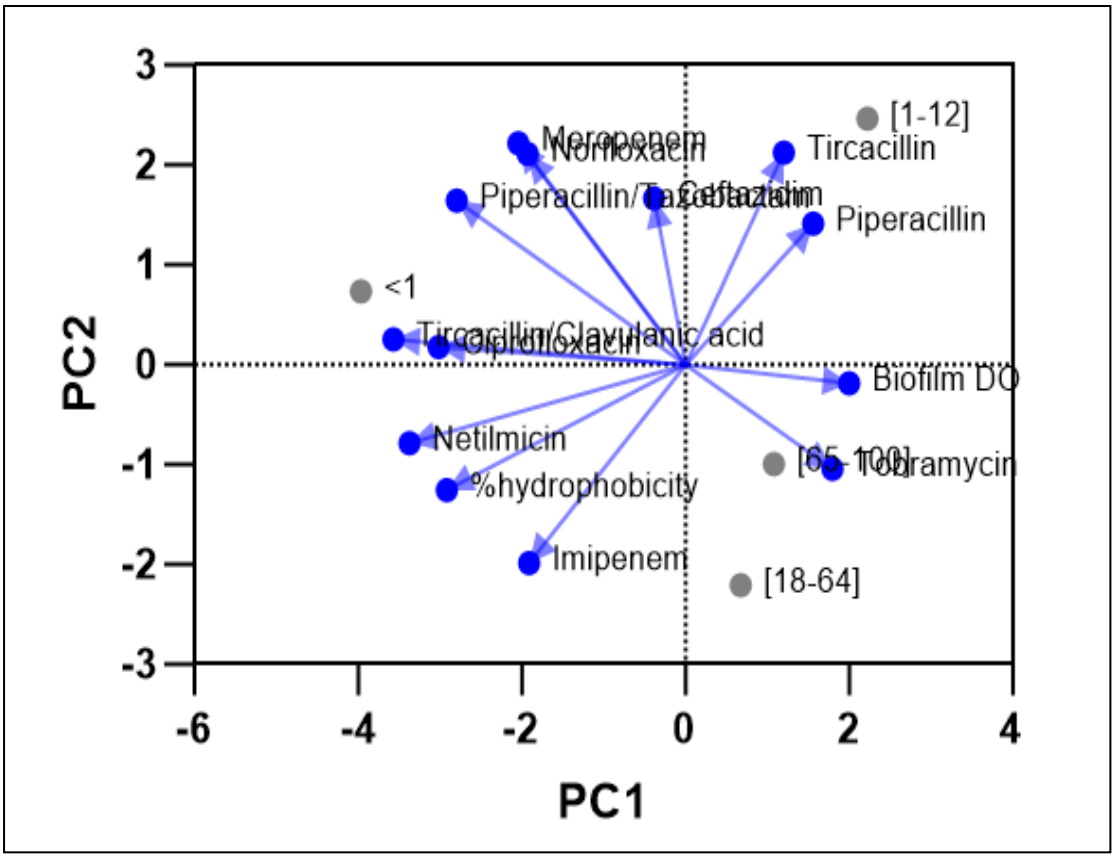

Figure 5: Correlation between antibiotic activities, hydrophobicity and biofilm formation using class ages

Figure 6 showed the correlation between biofilm formation, resistance index and hydrophobicity in the wards. A positive correlation was observed between biofilm production and multi-drug resistance. On the other hand, hydrophobicity was not linked to multi-drug resistance or biofilm formation. Among the wards, *P. aeruginosa* isolated from the intensivecare unit, medicine and external patients produced biofilms and had the highest resistance indexes.

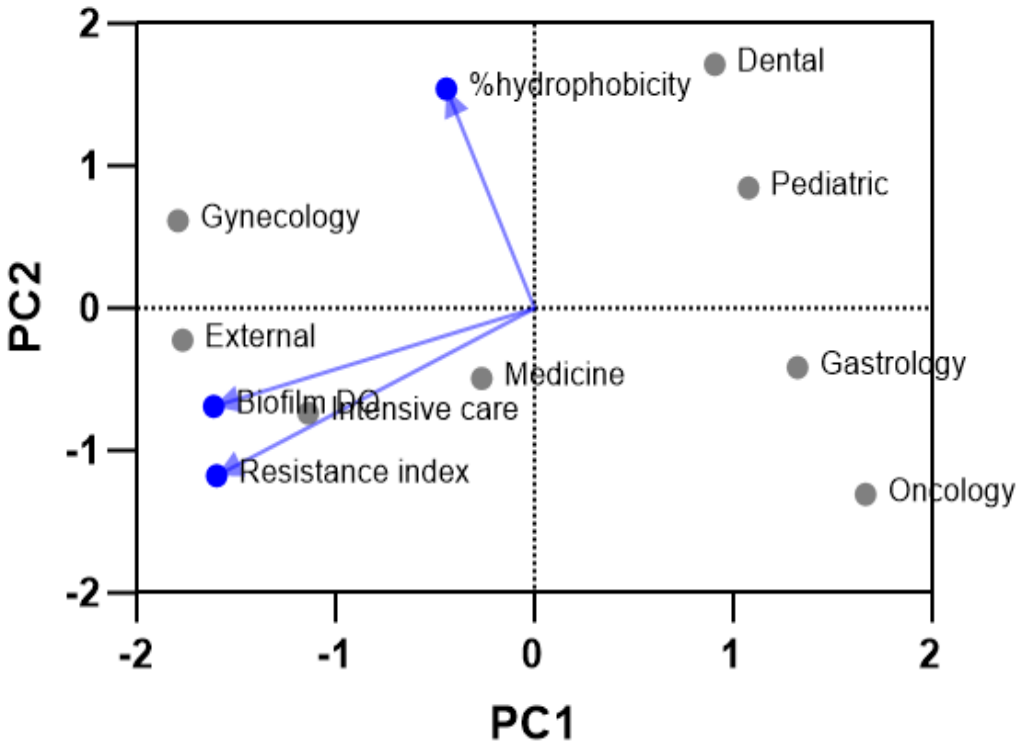

Figure 6: Correlation between hydrophobicity, resistance index and biofilm formation in wards

The study of biofilm formation, hydrophobicity and multi-drug resistance (figure 7) in the samples also revealed that the resistance index was positively correlated with biofilm production but not with hydrophobicity. Isolates from lochia and wounds were biofilm producers and showed multidrug resistance while isolates from urine and pus was very hydrophobic.

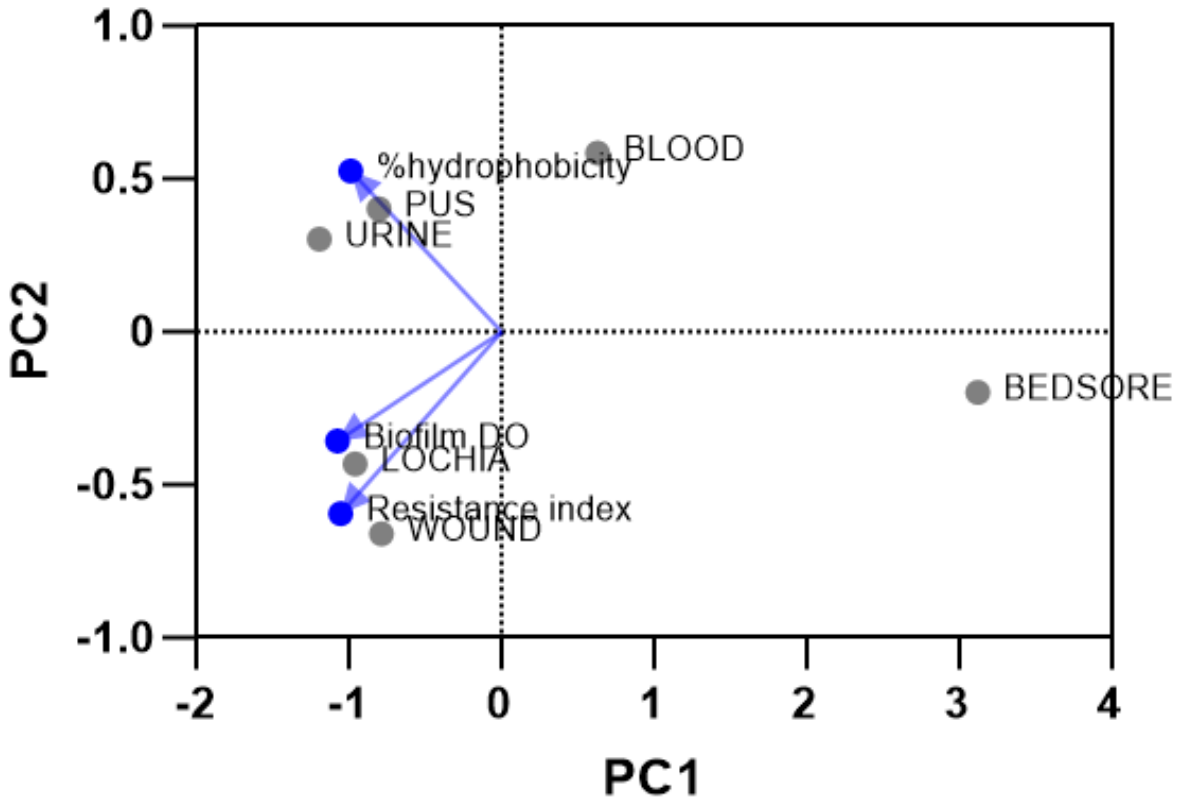

Figure 7: Correlation between hydrophobicity resistance index and biofilm formation among samples

DISCUSSION

The absence of surveillance committees in hospitals does not allow the possibility to have an up-to-date data on the factors that can influence the effectiveness of antibiotic treatment of *P. aeruginosa* infections in hospitals. Our study aimed to assess the impact of biofilms and hydrophobicity on the activity of *Pseudomonas aeruginosa*.

In this study, 300 participants were selected and the frequency of *Pseudomonas aeruginosa* was 10%. The majority of patients attending these health facilities were men (50.67%). These results are similar to those of Wang *et al.* at the National Taiwan University Hospital, who found that 64.9% of patients in hospitals were male, compared with 35.1% who were female[14]. Studies conducted by Piano *et al*. (2018) in America, Asia and Europe also revealed that men (69%) were more frequently in hospitals.

Patients of [18-64] and [64-100] consulted most frequently for an infection. This could be explained by the immune failure in elderly patients, which exposes them most to infections, resulting in their regular attendance at healthcare facilities. Studies conducted by Piano *et al*. also revealed that adults were most frequently encountered in healthcare settings [15].

The higher number of people observed in hospitals than at Centre Pasteur du Cameroun could be explained by the fact that the majority of patients are hospitalised and prefer to have their tests carried out directly in the laboratory of the hospital treating them. These patients go at the Centre Pasteur du Cameroun for biological analysis when the analysis requested by the physician is not available in the hospital where the patient is treated or if they are not hospitalised.

The most common sample was urine (35.33%), followed by pus (29.33%) and blood (20.67%). The predominance of these samples showed that the most frequent bacterial infections are urinary tract infections, purulent infections of superficial and deep organs, and septicemia, indicating that vital organs are affected and may be one of the causes of increased morbidity and mortality. Studies carried out by Piano *et al.* in hospitals of Europe, America and Asia also showed that the most common infections were purulent infections as spontaneous bacterial peritonitis (SBP; 27%), urinary tract infection (22%) and deep infections as pneumonia (19%)[15].

Pus and urine were the samples represented in most wards. This distribution supports the hypothesis that most patients received in the various wards for an infectious diagnosis

consult for a purulent infection or urinary tract infection. The most frequently requested bacteriological tests in outpatients was cytobacteriological test of urine (30.38%), followed by pus analysis (32.91%). This demonstrates that non-hospitalised patients were also presumed to have infections found in hospitalised patients.

In this study, principal component analysis was used to group antibiotics with similar activities according to health facilities. The activity of tircacillin increased with the activity of piperacillin/ Tazobactam; the activity of netilmicin increased with the activity of ciprofloxacin and that of ceftazidim; the activity of meropenem increased with the activity of norfloxacin and that of tircacillin/Clavulanic acid. These correlations reveal probable similarities between the activities of antibiotics from different families. UTHY isolates were more inhibited by Tircacillin and Piperacillin/Tazobactam, while GHY isolates were more inhibited by Imipenem and CHY isolates were more inhibited by Netilmicin, Ciprofloxacin and Ceftazidim. This difference in activity could be explained by the divergence in resistance profiles observed in the hospitals, demonstrating the need to establish a resistance monitoring committee in each hospital.

Pseudomonas aeruginosa isolates collected in this study were strong biofilm producers (30%), moderate biofilm producers (53.33%) and Non-biofilm producers (26.67%). These results are similar to those of Madaha *et al*. who found that 30.67% isolates of *P. aeruginosa* were strong biofilm producers while a higher proportion were moderate biofilm and non-biofilm producers in this study compared to her findings which showed 34.67% and 17.33% respectively [16]. Our results are differents from previous results which revealed that 16.25% produced strong biofilm, 33.75% moderate biofilm and 6.25% of isolates were non-biofilm producer [17]. As reported by Moradali *et al*. and Rehm *et al*., biofilm is one of the key strategies for the survival of species against unexpected changes of living conditions such as temperature and nutrient availability[18][19]. This virulence factor will therefore facilitate the persistence of *P. aeruginosa* in the environment over a long period of time, posing a risk of contamination in healthcare environments for new patients, visitors and healthcare staff. Previous studies also reported that the eradication of biofilm-forming *P. aeruginosa* is almost impossible in some infections and can lead to higher costs of treatment, longer periods of hospitalization and complications resulting in increased morbidity and mortality[20].

A positive correlation was observed between biofilm production and the resistance index. These results show that biofilm production promotes resistance to several antibiotics. Our results are similar to those of Abidi *et al.* who reported that biofilm production was significantly higher in MDR isolates in Pakistan [21]. Our results are different from the findings of Kamali *et al*. who showed that *P. aeruginosa* that produced biofilm and also those carrying biofilm-associated genes were mainly considered as non-MDR [17]. Although reported in previous studies that biofilm production is not necessarily correlated with multidrug resistance, biofilm production is an additional challenge when treating infections caused by MDR pathogens [20].

Previous studies have used hydrophobicity to predict bacterial adhesion. In this study, the hydrophobicity index of bacterial surfaces was measured by quantifying bacterial adhesion to xylene. Of the 30 isolates, 20% were highly hydrophobic, 23.33% moderately hydrophobic and 56.67% were low hydrophobic. This shows that some isolates of *P. aeruginosa* had a better ability to adhere to tissues than other isolates. Isolates from urine and pus were highly hydrophobic compared to isolates from other samples. Bacteria attach to tissue of humans in a highly selective manner and attachment is thought to be the first step leading to colonization and can facilitate the degradation of the patient's organs. This indicates a high risk of organ damage in patients with urinary tract or suppurative infections.

The activities of almost all the antibiotics increased when hydrophobicity decreased, with the exception of netilmicin and imipenem, whose activity increased despite the increase in hydrophobicity. The negative linear correlation observed between hydrophobicity and inhibition diameters demonstrates that hydrophobicity contributes to antibiotic resistance. The results support the hypothesis that hydrophobic interactions play a major role in *P. aeruginosa* adherence and subsequent plaque accumulation which will prevent antibiotics from reaching its target. This supports the hypothesis that an antibiotic contained in a lipid excipient could easily adhere to the bacterial target compared with an antibiotic contained in an aqueous excipient. Several studies have suggested that the hydrophobic properties of bacteria may be an important factor in their adherence to host tissue [22]. However, Hydrophobicity was not correlated with multidrug resistance or biofilm formation.

This study has some limitations. The study was conducted only in reference settings of Yaounde due to the unavailability of laboratory resources for bacteriological analysis in remote settings. This indicates serious data gaps in remote laboratories, particularly surveillance of bacterial infections, emphasising the need to expand microbiology laboratory capacity and data collection systems to improve our understanding [3]. The sample size should also be increased in future studies, including the number of hospitals and patients.

CONCLUSION

Strengthening the surveillance system and investigating the determinants of resistance are important for improving antibiotic selection and patient treatment. Our findings demonstrated that antibiotic resistance increase with biofilm production and hydrophobicity in *Pseudomonas aeruginosa*. The multidrug resistance was positively correlated with

biofilm production but not with hydrophobicity and patients from intensive care unit, medicine and external patients had the highest resistance indexes.

Competing interests

The author(s) declare that they have no competing interests.

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Ethical approval

This study was approved by the Ethical Committee of the Delegation of Public Health for the Centre Region of Cameroon under the approval number: 0191AAR/MINSANTE/DRSPL/BCASS. This evaluation was conducted according to the guidelines of the Declaration of Helsinki. To preserve the confidentiality of the participants, all laboratory samples and other patient documents were coded. All information obtained was confidential, participants' data were entered into the database and the access to this database was protected with a password.

Availability of data and materials

The datasets supporting the conclusions of this study are presented in this main paper.

Author's contributions

YLGNT conceived and designed the intellectual content and wrote the original manuscript; YBII designed the intellectual and reviewed the manuscript; EEL conducted the primary analysis, edited and reviewed the manuscript; SS edited and reviewed the manuscript; AN prepared figures and table; MM, CS, RN, MSDK reviewed the manuscript; HGK designed the intellectual content and substantively revised the manuscript; F-XE coordinated research activities and reviewed the manuscript. All authors have approved the final manuscript and agreed to be personally accountable for the author's own contributions.

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