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Amina Hoceini, Karima Benbaha, Hafidha Adoul, Ahlem Bensaber, Hichem Tahraoui, et al.. Evaluation of Biofilm Forming Potential and Antimicrobial Resistance Profile of *S. aureus* and *P. aeruginosa* Isolated from Peripheral Venous Catheters and Urinary Catheters In Algeria, in vitro Study. *Advanced Research in Life Sciences*, 2023, 7 (1), pp.83-92. 10.2478/arls-2023-0010 . hal-04261550

HAL Id: hal-04261550

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Submitted on 27 Oct 2023

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DOI: 10.2478/arls-2023-0010
Research Article

Evaluation of Biofilm Forming Potential and Antimicrobial Resistance Profile of *S. aureus* and *P. aeruginosa* Isolated from Peripheral Venous Catheters and Urinary Catheters In Algeria, in vitro Study

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Received June, 2023; Revised July, 2023; Accepted July, 2023

Abstract

Pseudomonas aeruginosa and *Staphylococcus aureus* are prominent pathogens responsible for device-associated infections that pose a significant challenge in clinical management. The biofilm-forming ability of these bacteria is a major factor contributing to treatment failures and the recurrence of infections. This study was conducted to evaluate the biofilm production potential of these bacteria isolated from urinary and peripheral venous catheters. Additionally, the study aimed to determine the antibiotic resistance pattern of biofilm producers and non-biofilm producers strains. The identification of bacteria followed conventional microbiological procedures, while the detection of biofilm production involved both qualitative and quantitative methods using Congo red agar (CRA) and Tissue Culture Plate (TCP) techniques, respectively. Antibiotic susceptibility was determined through the Kirby-Bauer disc diffusion method. Of the 22 strains of *P. aeruginosa* and 29 strains of *S. aureus* isolated from 116 Peripheral venous catheters and 20 urinary catheters, the CRA method identified 27.27% of *P. aeruginosa* and 24.14% of *S. aureus* strains as strong biofilm producers. Meanwhile, 9.09% of *P. aeruginosa* and 51.72% of *S. aureus* strains were moderate biofilm producers. The TCP method detected 9.09% of *P. aeruginosa* and 37.93% of *S. aureus* strains as strong biofilm producers, while 54.55% of *P. aeruginosa* and 48.28% of *S. aureus* strains were moderate biofilm producers. The antibiotic susceptibility test revealed that biofilm-producing bacteria were mostly more resistant to antibiotics than non-biofilm producers. All *S. aureus* strains, were resistant to Penicillin (100%), but susceptible to Vancomycin (100%) and Teicoplanin (100%). All *P. aeruginosa* strains were susceptible to almost all groups of antibiotics, except for Ticarcillin (18.18%), Piperacillin (27.27%), Imipenem (100%), and Rifampicin (100%). Our findings showed that biofilm-producer strains exhibited high resistance rates to only some antibiotics, and emphasized the importance of seeking new strategies to prevent biofilm formation and combat the emergence of antibiotic resistance.


Keywords: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, biofilm formation, antibiotic resistance, catheters.

Introduction

Medical facilities increasingly rely on medical devices and prostheses to provide optimal patient

care, and these devices have become an integral part of modern medicine. Nosocomial infections frequently linked to the use of medical devices can result in complications that can prolong hospital stays and increase patient morbidity and mortality rates among patients. Biofilms play an important role in medical-device associated infections. These biofilms are complex

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communities of microorganisms that adhere to surfaces and are enclosed within a protective extracellular matrix composed of a combination of exopolysaccharides (EPS), environmental DNA (eDNA), proteins, surfactants, lipids, and water [1] [2]. This matrix makes it difficult for antimicrobial agents to penetrate and kill the microorganisms [3].

Quorum sensing is a mechanism of communication between microbial cells that controls specific processes, such as biofilm formation. Biofilm growth is associated with quorum-sensing-regulated mechanisms and an increased frequency of mutations [4], which can lead to the development of antibiotic-resistant strains of bacteria.

Medical devices and prostheses provide essential support and care to patients, and can serve as breeding grounds for bacteria colonization. Among the most commonly found bacteria in medical devices are *Enterococcus faecalis*, *Staphylococcus aureus* (*S. aureus*), *S. epidermidis*, *Streptococcus viridans*, *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* (*P. aeruginosa*) [5]. Furthermore, the interaction between different bacterial species can further complicate the biofilm formation. For instance, co-colonization of *S. aureus* and *P. aeruginosa* increase biofilm production and induce the differential expression of a significant number of genes involved in immune response, inflammation, signaling, development, and defense [6]. Hence, a thick coats of exopolymeric matrix protects biofilm cells from phagocytosis [7].

Detecting and managing biofilm formation early is crucial to develop effective treatments. Early and aggressive antibiotic prophylaxis or therapy can prevent biofilm formation, while chronic suppressive therapy can be used to treat it [4]. Furthermore, understanding the mechanisms of biofilm formation, including quorum sensing and gene expression, can help develop new strategies for biofilm prevention and managing [4].

Biofilm formation is a major challenge in the management of infections associated with medical devices. Therefore, the investigation of the ability of *S. aureus* and *P. aeruginosa* strains obtained from medical devices to form biofilms using different methods is essential. The most commonly used are the tissue culture plate (TCP) [8], Congo red agar (CRA) method [9] and the tube method (TM) [10]. The microtitre-plate technique (TCP) is a quantitative method based on measuring the optical density (O.D) of stained bacterial biofilms found on the bottom of tissue culture plates [11], while the qualitative CRA plate

test is based on a subjective chromatic evaluation [9].

Moreover, *S. aureus* and *P. aeruginosa* exhibited high levels of antibiotic resistance probably occurred by the protective action of β -lactamases in impairing the penetration of β -lactams in the biofilm [12]. A resistance of Gram-negative bacilli such as *P. aeruginosa* to carbapenems can be related to the decreased permeability of the bacterial outer membrane and hyperexpression of efflux pumps [12]. Understanding the antibiotic susceptibility profiles of these strains will provide insights into the choice of appropriate antimicrobial agents for treatment.

A recently published report of the Antimicrobial Resistance (AMR) in the WHO African Region including Algeria have revealed the need to more timely and effective surveillance studies and programmes for bacterial infections to deal with the current AMR threats presented [13]. Moreover, the lack of studies on microbial biofilms and AMR in our region, the misuse of antibiotics by most people and the lack of antibiotic resistance surveillance laboratory made it necessary to elaborate this study.

Overall, this study aims to provide a comprehensive understanding of the ability of *S. aureus* and *P. aeruginosa* strains obtained from medical devices to form biofilms and their antibiotic resistance patterns. The findings of this study could contribute to developing effective strategies for preventing and treating biofilm-associated infections in healthcare setting.

Materials and Methods

Samples collection

Between October 2018 and August 2019, an extensive collection of 136 catheters was gathered from six diverse medical departments, including maternity, pediatrics, emergency, internal medicine, gynecology, and oncology, in the Public Hospital Establishments of Berrouaghia, Medea, Beni Slimane and Sidi Naamane (Algeria). All catheters were carefully collected in sterile glass tubes under strict aseptic conditions to prevent any contamination and were promptly delivered to the Microbiology laboratory within a maximum of two hours to ensure the validity and reliability of the samples.

Sample processing

The research was conducted at the Faculty of Sciences and the microbiology laboratory of SAIDAL Médéa in Algeria. Following the removal of medical devices, a microbiological analysis was performed using the "Brun-Buisson" technique [14]. The distal end of each device was aseptically

cut, inoculated into Brain Heart Infusion Broth (BHIB), and vortexed for one minute before being incubated overnight at 37°C. *S. aureus* and *P. aeruginosa* were isolated on Chapman agar and Cetrimide agar, respectively. The isolates were identified based on standard microbiological procedures that included colony morphology, Gram's staining, and rapid tests (catalase, oxidase, coagulase, etc.). Biochemical characterization was performed using standard tests such as urease, nitrate reduction, Voges-Proskauer, citrate, indole, and triple sugar iron tests [15].

Biofilm Detection Methods

Two methods were employed to detect the presence of biofilms in the collected medical devices.

1. Congo Red Agar method (CRA)

To investigate slime production, CRA medium was used in accordance with Freeman et al. [9]. The medium was prepared by adding 37 g/L of BHIB, 50 g/L of sucrose, 10 g/L of agar, and 0.8 g/L of Congo Red indicator. The strains were suspended and inoculated in the medium and then incubated at 37°C for 24 hours. The strains that produced slime gave rise to black colonies, whereas red colonies indicated the absence of slime production. Strains that showed varying phenotypes, such as colonies with black centers and red outlines or red centers and black outlines, were considered moderately biofilm-forming.

2. Tissue culture plate method (TCP)

The Christensen et al. [10] method is considered the standard for detecting biofilms quantitatively. This method involves a colorimetric test after washing, staining, and destaining sessile cells using crystal violet or safranin in tubes or microtiter [16].

The strains were first grown and incubated overnight in Brain Heart Infusion (BHI) medium. The wells of a sterile 96-well microplate (polystyrene) were filled with 200 µL of the strain suspension adjusted to a final optical density of 0.1 at 600 nm. Sterile broths were used as blanks to ensure sterility and non-specific binding of the media. The microplates were then sterile-coated and incubated for 24 hours at 37 °C. After incubation, the plates were gently tapped to remove free bacteria and then washed with PBS (pH 7.3) four times.

Bacterial cells that formed a biofilm were stained with 0.1% (w/v) crystal violet. The crystal violet solution was then removed, and the wells were washed three times with deionized water and allowed to dry. The wells were then refilled with 200 µL of ethanol solution (95%), and the optical density (OD) of stained adherent biofilm was obtained using a micro ELISA autoreader (bioTek,

Germany) at 600 nm. The experiment was performed in triplicate and repeated three times. According to Stepanovic et al. [11], the interpretation of biofilm production was divided into four categories: no biofilm producer (OD < OD_c), weak biofilm producer (OD_c < OD < 2xOD_c), moderate biofilm producer (2xOD_c < OD < 4xOD_c), and strong biofilm producer (4xOD_c < OD), where the cut-off absorbance (OD_c) was determined as the average OD of the negative control plus three times the standard deviation of the negative control. The average OD values and standard deviations were calculated using Excel software.

Antibiotic susceptibility test

The antibiotic susceptibility test was conducted to evaluate the susceptibility of biofilm-producing bacteria on Mueller Hinton agar (Oxoid, UK) using the Kirby-Bauer disc diffusion technique following the guidelines set by the Clinical and Laboratory Standards Institute (CLSI) [17]. All antibiotic discs used in the test were sourced from Oxoid, UK.

All identified staphylococcal strains were subjected to antibiotic susceptibility testing, which involved the use of various antibiotic discs, including Penicillin (10 IU), Oxacillin (1 µg), Cefoxitin (30 µg), Amikacin (30 µg), Gentamicin (10 µg), Kanamycin (30 µg), Erythromycin (15 µg), Clindamycin (2 µg), Pristinamycin (15 µg), Ofloxacin (5 µg), Chloramphenicol (30 µg), Vancomycin-Teicoplanin (30 µg), Tetracycline (30 µg), Trimethoprim+sulfamethoxazole (1.25/23.75 µg), Fusidic acid (10 µg), and Fosfomycin (50 µg). Similarly, for *P. aeruginosa*, the antibiotic susceptibility test involved the use of 14 different antibiotic discs, including Ticarcillin (75 µg), Ticarcillin + clavulanic acid (75/10 µg), Piperacillin (100 µg), Ceftazidime (30 µg), Aztreonam (30 µg), Imipenem (10 µg), Amikacin (30 µg), Gentamicin (10 µg), Tobramycin (10 µg), Netilmicin (30 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Rifampicin (30 µg), and Colistin (10 µg).

Results and discussion

This research collected 136 catheters aseptically from six services of the Public Hospital Establishments of Berrouaghia, Medea, Beni Slimane and Sidi Naamane (Algeria). The catheters included 116 Peripheral venous catheters and 20 urinary catheters. The majority of samples were obtained from the Oncology department (33.09 %), followed by the Maternity department (26.47 %), the department of Gynecology (22.80 %), the emergency unit (10.29 %), the Pediatric department (5.88 %), and the internal medicine service (1.47 %). Among the

collected samples, 109 (80%) showed positive culture results. Interestingly, previous research conducted in a hospital in Tangerang, Indonesia, showed microbial growth in 78% (85) of catheter samples [18]. The high percentage of positive cultures is probably related to the use of inadequate skin antiseptic before the catheters insertion, which increased the risk of subsequent migration of skin bacteria along the catheter surface. Furthermore, the duration of catheterization of patients involved in this study is the most important factor in the development of the polymicrobial infections [18].

The study showed that most catheters (73) had only one type of microorganism present, including 52 catheters with *Staphylococcus* and 21 with *Pseudomonas*. However, 36 catheters had both *Staphylococcus* and *Pseudomonas* strains. Our findings are consistent with other studies that have reported that most catheters had a single microorganism present, including *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. epidermidis*, *Candida spp.*, *S. haemolyticus*, and *S. aureus*. And only Four catheters had more than one microorganism detected, including *E. faecalis* and *S. epidermidis*, *P. aeruginosa* and *S. aureus*, *Proteus spp.* and *S. epidermidis*, and *E. coli* and *P. aeruginosa* [19]. Additionally, a study conducted by Gunardi *et al.* [18] reported that 62.8% of the catheter cultures had a single microorganism, while only 26.1% and 11.1% were polymicrobial with two and three microorganisms, respectively.

Based on the identification approach of the isolated staphylococci and *Pseudomonas* strains, we isolated 29 strains of *S. aureus* and 22 strains of *P. aeruginosa*. Interestingly, the findings

revealed that coagulase-negative staphylococci (CNS) (*S. epidermidis*, *S. lentus*, *S. xylosus*, *S. auricularis* and *S. cohnii*) were the most frequently isolated strains from catheters compared to *S. aureus* isolation (Figure 1). The CNS are part of the commensal bacterial microflora of the human body that can cause a catheter infection by its introduction from the skin during device insertion [20]. Previous research findings revealed that *S. epidermidis* and other CNS are usually the most common causative microorganisms in device-related infections [20]. A study conducted in Algeria showed that *Klebsiella pneumoniae* (26.5%), Coagulase Negative Staphylococci (23.5%), and *Staphylococcus aureus* (23.5%) predominantly caused central venous catheter (CVCs) related infections [21]. Furthermore, research conducted by Zhang *et al.* [22] indicated that *Staphylococcus epidermidis* (11.84%) and *Staphylococcus aureus* (5.29%) were the primary Gram-positive bacteria, whereas *Pseudomonas aeruginosa* (11.59%) and *Acinetobacter baumannii* (8.82%) were the predominant Gram-negative bacteria isolated from CVCs obtained from the Intensive Care Unit (ICU) of Qianfoshan Hospital in China. Another study reported that *Klebsiella pneumoniae* (10%) and *Pseudomonas aeruginosa* (7%) were the most common Gram-negative bacteria isolated [23]. *Pseudomonas aeruginosa* is an environmental bacterium widely resistant to antibiotics and is also considered an opportunistic pathogen responsible for nosocomial infections in hospitalized patients [24]. Therefore, it is crucial to implement aseptic techniques during catheter insertion to enhance patient outcomes and overall quality of care.

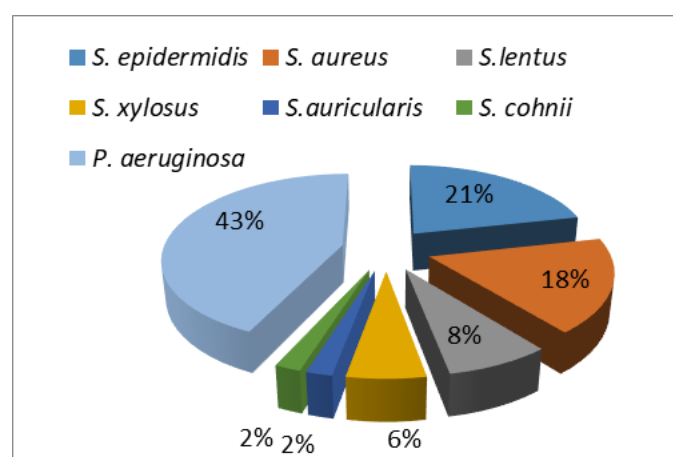


Figure 1. Distribution of *P. aeruginosa* and staphylococci isolated from catheters

The detection of biofilm formation by CRA method
This qualitative method is based on the phenotypic character of the strains shown on

Congo Red Agar. 22 strains of *P. aeruginosa* and 29 strains of *S. aureus* isolated were tested to

highlight their capacity to form biofilm by CRA techniques.

Investigation of slime production on CRA revealed that 30 strains (08 *Pseudomonas aeruginosa* and 22 *Staphylococcus aureus*) were slime forming (strong and medium), representing 75.86 % of

Staphylococcus aureus strains and 36.3 % of *Pseudomonas aeruginosa* strains. While the other isolates were non-biofilm forming (14 *Pseudomonas aeruginosa* and 7 *Staphylococcus aureus*) (Figure 2)..

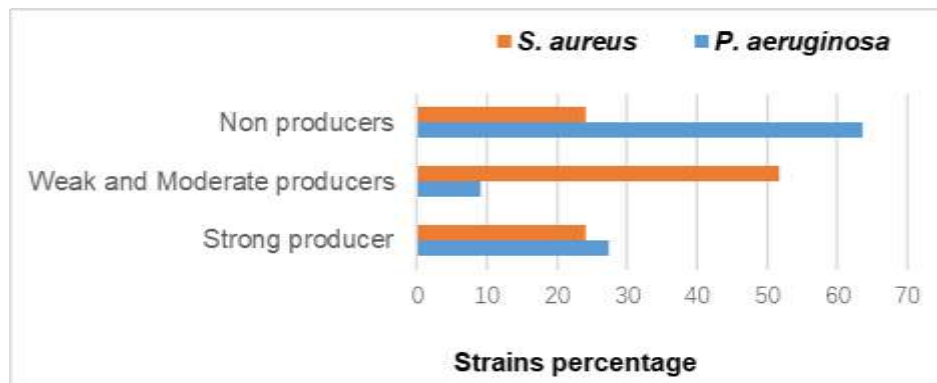


Figure 2. Biofilm forming capacity by RCA technique

Our study results align with previous research findings, which indicate that the majority of *S. aureus* strains isolated (60.8%) are capable of producing biofilms using Congo Red agar culture [25]. Similar results (67.9%) were also reported by Kara Terki *et al.* [26]. In contrast, studies conducted by Knobloch *et al.* [27], Mathur *et al.* [28], and Taj *et al.* [29] revealed that only a small percentage of Staphylococci were capable of producing biofilms (3.8%, 5.3%, and 3.4%, respectively). This disparity in results may be attributed to the differences in the preparation techniques used for RCA.

Furthermore, the use of indwelling catheters is associated with a 14-fold increase in the risk of infection occurrence [30]. The Polysaccharide intercellular adhesin (PIA), which is synthesized by the *ica* locus, is the primary factor in staphylococcal colonization of medical equipment [31]. The production of PIA depends on environmental conditions [26] and plays a crucial role in bacterial cell adhesion, biofilm matrix architecture, bacterial adhesion to biomaterial surfaces, and evasion from host immune responses [32].

Additionally, a study found that 90% of *P. aeruginosa* strains isolated from urinary catheter tips, intravenous catheter tips, and other samples were strong biofilm producers [33]. The strong biofilm formers were shown to promote the expression of the *ica* gene of *S. aureus*, and the

cup A gene of *P. aeruginosa* [34]. Moreover, biofilm formation of *P. aeruginosa* isolate is facilitated by the production of extracellular DNA (eDNA), rhamnolipids, lectins, elastases, and toxins [35].

Overall, our findings highlight the importance of effective strategies to prevent and control biofilm formation on medical devices, particularly in patients with indwelling catheters, to improve patient outcomes and reduce the risk of infection occurrence.

Quantification of biofilm-producing capacity by TCP method

Figure 3 displays the results of the Microtitre Plate assay used to determine the biofilm production ability of 51 strains. It is noteworthy that the TCP quantitative method yielded different outcomes compared to the qualitative method (CRA). The data revealed that 37.93% (n=11) of *S. aureus* and 9.09% (n=2) of *P. aeruginosa* were categorized as strong biofilm producers, while 48.28% (n=14) of *S. aureus* and 54.55% (n=12) of *P. aeruginosa* were considered as weak and moderate producers. On the other hand, 13.79% (n=4) of *S. aureus* and 36.36% (n=8) of *P. aeruginosa* were non-biofilm producers. These results emphasize the importance of employing accurate and reliable techniques for the detection and evaluation of biofilm production ability of bacterial strains.

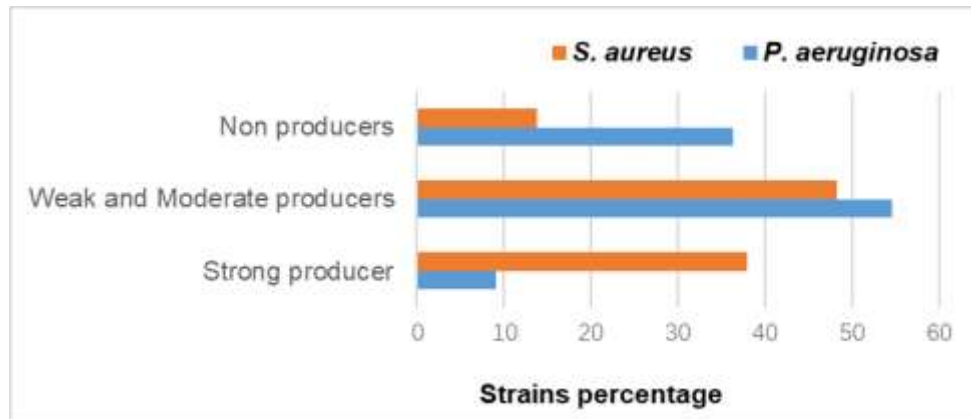


Figure 3. Biofilm producing capacity of isolated strains by the TCP Method

The TCP method revealed that a high proportion of *S. aureus* (86.21%) isolated in our study were biofilm producers. This finding is consistent with another study by Fatima *et al.* [36], which reported a similarly high percentage (64.89%) of *S. aureus* biofilm producers. In contrast, Kara Terki *et al.* [26] found that only 32.1% of their 28 *S. aureus* strains produced biofilm in BHIB medium. These results agree with other studies showing that only a few strains form biofilm in growth media without supplements such as sugar [37]. In a separate study, 149 *P. aeruginosa* isolates from urinary catheters in St. Anne's University Hospital were evaluated, with 20% classified as non-biofilm producers, 9.4% as weak producers, 14.1% as intermediate producers, and 74.5% as strong

producers. In contrast, all 15 strains of *S. aureus* in the same study were classified as strong biofilm producers [38]. A previous study has shown that a conformational change in the LasR protein structure might be the reason of decreasing biofilm ability for *P. aeruginosa* [39].

Antibiotic resistance

The antibiotic sensitivity profile for both biofilm producing and non-producing *P. aeruginosa* and *S. aureus* is presented in figures 4 and 5, respectively. Forming biofilms on catheters poses a significant challenge for treating infections because the biofilm structure provides a protective shield against antibiotics, making the bacteria within the biofilm more resistant to treatment.

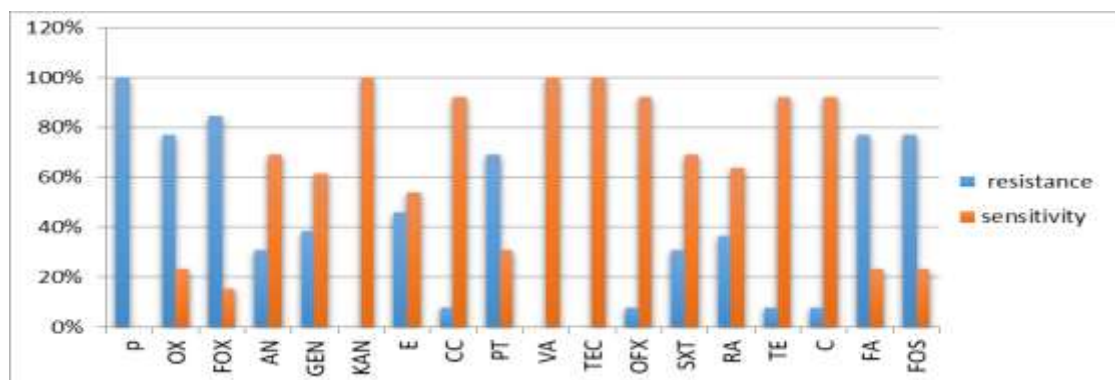


Figure 4. Antibiotic resistance profile of *S. aureus*



Figure 5. Antibiotic resistance profile of *P. aeruginosa*

The antibiotic resistance patterns of Penicillin (P), Oxacillin (OX), Cefoxitin (FOX), Amikacin (AN), Gentamicin (GEN), Kanamycin (KAN), Erythromycin (E), Clindamycin (CC), Pristinamycin (PT), Vancomycin (VA), Teicoplanin (TEC), Ofloxacin (OFX), Trimethoprim/sulfamethoxazole (SXT), Rifampicin (RA), Tetracycline (TE), Chloramphenicol (C), Fusidic acid (FA), and Fosfomycin (FOS) were compared between biofilm producers and non-producers of *S. aureus*. The study showed that biofilm producers exhibited higher resistance rates to rifampicin, Pristinamycin, Trimethoprim/sulfamethoxazole, and Erythromycin. The findings from Sabir *et al.* [40] corroborated our results, which showed that all strains were resistant to Penicillin (100%). However, no resistance was observed towards Vancomycin and Teicoplanin in biofilm producers and non-producers of *S. aureus*. Previous studies have reported 100% sensitivity of *S. aureus* strains to vancomycin [41] which is strongly related to low usage of these antibiotics due to their high costs.

Our study found that 84.6% of our isolates were resistant to Cefoxitin, while 46.15% and 38.46% were resistant to Erythromycin and Gentamicin, and the results were consistent with previous findings [41]. We also observed high rates of resistance towards Cefoxitin, Oxacillin, Fusidic acid, Fosfomycin, and Pristinamycin at 84.61%, 76.92%, 76.92%, 76.92%, and 69.23%, respectively. In contrast, we observed low resistance rates towards Clindamycin (7.69%), Ofloxacin (7.69%), Tetracycline (7.69%), and Chloramphenicol (7.69%). Some of these antibiotics are commonly used for preliminary treatment of *S. aureus* infections.

The growth of antibiotic resistance is linked to many factors such as self-medication without prescription and the misuse of antibiotics in the community, animals, agriculture and the

environment. Moreover, the high rates of antibiotic resistance observed among biofilm producers may be attributed to the difficulty of antibiotics penetrating the biofilm, the slow growth rate of bacteria, and antibiotic degradation mechanisms [42]. Additionally, biofilm formation promotes horizontal transfer of genes coding for drug resistance and other virulence factors [43].

In our study, both biofilm-producing and non-producing *P. aeruginosa* strains showed susceptibility to nearly all of the antibiotic groups used, except for TIC, PI, IMI and RA, for which high resistance rates were observed in biofilm-producing strains. Despite these findings, Cepas and his collaborators have demonstrated no statistically significant relationship between multi-drug resistant *P. aeruginosa* and biofilm formation [44].

However, earlier research has shown resistance to several antibiotics and a high susceptibility to colistin [45]. The high susceptibility to colistin is attributed to its limited use as a last resort treatment against serious MDR infections because of its nephrotoxicity caused by the oxidative stress and the activation of inflammatory and apoptotic pathways particularly in renal proximal tubules [46].

Cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones are four crucial groups of anti-pseudomonal agents [47]. However, the overuse of antibiotics and prior exposure to a specific antibiotic is not only linked to the emergence of resistance to that antibiotic but also to antibiotics belonging to different classes [48]. Studies have demonstrated that prior use of fluoroquinolones or carbapenems were independent risk factors for resistance to ciprofloxacin and imipenem, and prior use of extended-spectrum cephalosporins was a risk factor for piperacillin resistance [49]. Furthermore, in some cases, horizontal gene transfer has been found to be the primary mechanism of acquisition

of antibiotic resistance [50].

Conclusions

Antibiotic resistance has become a pressing concern, as it is not limited to a specific antibiotic but can spread to different classes of antibiotics. The study's results indicate the high resistance rates of biofilm-producing strains of *P. aeruginosa* and *S. aureus* to multiple groups of antibiotics, underscoring the need for innovative approaches to control biofilm formation and improve patient management. The overuse and misuse of antibiotics have been identified as the main drivers of antibiotic resistance, which ultimately reduces the efficacy of current treatments. Therefore, it is imperative to implement infection control measures and strategies to prevent biofilm formation in healthcare facilities, which can reduce the incidence of catheter-related infections and decrease the dependence on antibiotics. The study's findings demand immediate action from healthcare professionals to develop and implement effective strategies to combat antibiotic resistance and prevent its spread. A coordinated effort is necessary to ensure effective treatment options for future patients while preserving the effectiveness of antibiotics.

The limitations of this study are the lack of detailed information of the patients, conditions, treatments, antiseptic used, type of catheters, and the exact duration and reason of catheterization for each patients. Hence, it is necessary to improve the electronic record of laboratory sample information to promote future research in this field. Moreover, further studies of risk factors should be encouraged to guide clinical practice.

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