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ORIGINAL ARTICLE

Study of Antibiotic Resistance and Biofilm Formation of Staphylococci Isolated from Dental Department Liquid Effluents

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KEYWORDS

Staphylococcus capitis;
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ABSTRACT: *Staphylococcus* sp. are pathogenic bacteria commonly found in clinical environments, including dental clinics. Their pathogenic potential derives from their ability to express various virulence factors. Our study was conducted to investigate the prevalence of antibiotic resistance and biofilm formation by strains of Staphylococcus isolated from the liquid effluent of two (02) dental practices in Sidi Bel Abbés, Algeria. Seven strains (07) were purified from three (03) oral rinse samples collected. Three (03/07) were positive on Chapman agar, with positive catalase, confirming their belonging to the Staphylococcus genus. Biochemical gallery identification revealed three different biotypes: 4104100: *Staphylococcus capitis*, 6310111: *Staphylococcus warneri*, 6104100: *Staphylococcus capitis*, using the API STAPH system. All strains tested positive for free coagulase in tubes and bound coagulase, which the Staphytect Plus latex agglutination test confirmed. In addition, our strains showed positive DNase activity. The isolated strains were multi-resistant to antibiotics, with antibiotic susceptibility tests revealing total resistance (100%) to the following antibiotics: penicillin, oxacillin, cephalotin and nalidixic acid. The 03 strains also showed morphological characteristics of biofilm formation when grown on Congo red agar, in which biofilm-producing colonies appeared black. The study highlights the need for further research to explore the underlying resistance mechanisms and identify innovative strategies to prevent and eradicate biofilms to curb the spread of resistant bacterial strains.

INTRODUCTION

Staphylococcus sp. are pathogenic bacteria commonly found in clinical environments, including dental clinics [1]. Their pathogenic potential stems from their ability to express a wide variety of virulence factors. Among these, there are genes involved in the formation of sessile bacterial communities known as biofilms [2]. This biofilm formed by *Staphylococcus* sp. can lead to persistent infections, as they resist antimicrobial agents [3]. Biofilm in dental clinics forms in dental unit waterlines, posing infection risks, especially to immunocompromised patients [4]. These clinics use water to rinse tooth surfaces during various operations

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and to rinse the patient's mouth [5]. In addition, patient material such as blood and saliva is re-aspirated into the dental unit's water system, giving oral microorganisms the opportunity to colonize aquatic biofilms; these biofilms will act as a reservoir for ongoing bacterial contamination of the effluent water [6].

The aim of this study was therefore to evaluate the formation of biofilms by resistant *Staphylococcus* sp. isolated in a dental clinic. These biofilms act as a reservoir for ongoing bacterial contamination of effluent.

MATERIALS AND METHODS

Sampling and isolation

Three samples of oral rinsing fluid were taken from two dental clinics in Sidi Bel Abbès, Algeria. After culture in nutrient agar and Heart-Brain broth, bacterial strains were isolated on Chapman medium and incubated at 37°C for 24 to 48 hours. Four strains with distinct morphological characteristics were purified based on cultural traits and Gram staining and preserved on inclined nutrient agar. A series of biochemical tests was used to identify the strain. Initially, a macroscopic study of the colonies was carried out. This was followed by a microscopic study (gram staining) to determine cell morphology [7]. To assess the existence of the enzyme catalase, bacterial colonies were subjected to interaction with hydrogen peroxide [8]. The liberation of oxygen bubbles signifies a positive outcome [9].

Identification of staphylococci isolated by the API system

Complete biochemical identification was done using the API STAPH system. This standardized system comprises 20 microtubes containing dehydrated substrates, enabling 19 biochemical tests [10]. After inoculation with a bacterial suspension adjusted to 0.5 McFarland standards, the gallery was incubated at 37°C for 24 hours. Biochemical reactions were interpreted according to the manufacturer's instructions, generating a seven-digit code number. This code was then compared with the API database to identify the bacterial species [11].

Staphylocoagulase Activity

The free coagulase test was performed by mixing 0.5 ml bacterial suspension from overnight culture broths with 0.5 ml rabbit plasma incubated at 37°C and observing clot formation every 30 minutes, followed by a final reading after 24 hours for negative cases at 4 hours [12].

Staphytect Plus latex agglutination test

The Staphytect Plus, a latex agglutination test, was used to detect bound coagulase (clumping factor), protein A, and capsular polysaccharides, surface markers specific to *Staphylococcus aureus* [13].

Five colonies of each strain, grown on nutrient agar for 18 to 36 hours, were emulsified with one drop of test reagent (blue latex sensitized to porcine fibrinogen and rabbit IgG) and one drop of control reagent (non-sensitized latex) on a reaction card. After rotation for 20 seconds, agglutination of the sensitized latex was considered a as a positive result [14].

DNase assay

A DNase test was performed after subculturing the strain to be studied in BHIB, incubating for 18 hours at 37°C, inoculating using a heavy inoculum, and performing a central sting to detect the production of deoxyribonuclease, an enzyme that hydrolyzes DNA. The formation of a clear zone around the due colony identified DNase-producing colonies [15].

Antibiotic susceptibility testing

The susceptibility of strains to antibiotics was determined by the diffusion method on 4 mm thick Mueller-Hinton agar, by the recommendations of the Antibiogram Committee of the French Microbiology Society (CA-SFM). Pre-incubated cultures were diluted in the sterile buffer. Twelve antibiotics were tested:

Penicillin (PG-10 IU), Oxacillin (OX- 1 µg), Erythromycin (E-15 µg), Trimethoprim + Sulfamethoxazole (SXT- 1.25 / 23.75 µg), Tetracyclines (Te -30 µg), Vancomycin (Va-30 µg), Clindamycin (Cm-2 µg), Ciprofloxacin (CIP-5ug), Cephalotin (KF 30 ug), Nalidixic acid (Na 30ug), Gentamicin (GN-10ug), Chloramphenicol (C-30ug). After incubating the

antibiotic discs at 37°C for 18-24 h, measure the precise area around each disc in millimeters [16]. The reference strain *Staphylococcus aureus* ATCC 25923 was used for quality control of each antibiogram.

Biofilm formation test

Evaluate strains' ability to form biofilms. After cultivation of staphylococcal isolates on Congo red agar (CRA) and incubation for 24 hours at 37°C [17]. The formation of black colonies indicates a positive result for biofilm production, while the formation of red colonies indicates a negative result.

RESULTS AND DISCUSSION

Water in dental clinics is a preferred vector for various pathogenic microorganisms. This situation poses significant health risks for patients and dental practitioners [18]. Among these microorganisms, the coagulase-negative staphylococci threaten the environment by forming hospital strains with increased resistance and virulence, leading to prolonged circulation in nosocomial environments, particularly dental clinics [19]. This study investigated the prevalence of antibiotic-resistant staphylococcal biofilms in liquid effluents from dental services.

Sampling and isolation

Seven strains were purified from the three oral rinse samples collected from two dental clinics. Three of them were positive on Chapman agar, indicating the presence of staphylococci. After 24 hours of incubation, suspected staphylococci colonies on Chapman agar had the following characteristics: a yellow appearance, a smooth, round shape, a diameter between 1 and 2 mm, and yellow pigmentation [20]. All three isolated strains tested positive for the catalase test, confirming that they belong to the *Staphylococcus genus* [21].

Identification of staphylococci isolated by the API system

Identification by biochemical galleries revealed three different biotypes: 4104100: *Staphylococcus capitis*, 6310111: *Staphylococcus warner*, 6104100: *Staphylococcus capitis*

The Analytical Profile Index (API) tests facilitated accurately identifying microbial strains [22]. They successfully detected *Staphylococcus capitis*, corroborated by Mihai et *al.* (2020). They also identified *Staphylococcus warneri*, further validating their efficacy in recognizing this species among isolated strains [23].

Staphylocoagulase Activity

All three strains (03/03) also tested positive for coagulase, resulting in the appearance of a clot (Figure 1). It should be recognized that the coagulase gene is not exclusive to *S. aureus*. This gene can also be detected in other Staphylococcus species [24]. The coagulase is considered to be the most important virulence factor that coagulates plasma by conformational activation of prothrombin., transforming the fibrinogen into fibrin and then leading to the formation of abscesses and the persistence of microorganisms in the host tissue [25].

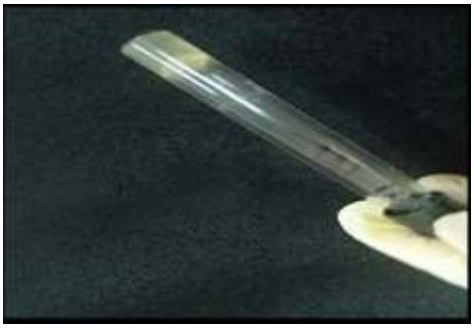

Figure 1. Positive coagulase test.

DNase assay

All the strains isolated showed positive DNase activity (Figure 2). Coagulation-negative staphylococci utilize DNase to break down DNA in the extracellular environment, enhancing their capacity to avoid immune system detection [26]. Furthermore, DNase contributes to pathogenicity through tissue degradation and nutrient provision while also playing a crucial role in biofilm formation, which confers resistance against host defenses and antibiotics [27]. Its prevalence in clinical isolates underlines its importance and complicates treatment strategies [26]. Extracellular DNA in wastewater can play an important role in the horizontal transfer of antimicrobial resistance genes. Exogenous DNA poses a risk to public health as it facilitates the spread of antibiotic resistance among bacteria, particularly in wastewater [28].

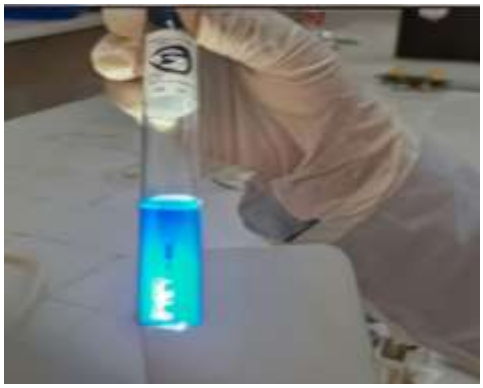

Figure 2. Positive Dnase test

Staphytect Plus latex agglutination test

All the strains isolated showed a positive result in the Staphytect Plus test, indicating the production of bound coagulase (Figure 3). This test is used to identify strains of *Staphylococcus aureus* by detecting clumping factor and protein A, which are generally present in coagulase-positive strains [7]. Nevertheless, ongoing updates on coagulase-negative staphylococci species have revealed a heterogeneous group, ranging from non-pathogenic to facultative pathogenic species, with distinct levels of potential virulence [29]. One of the characteristics that distinguishes pathogenic from less pathogenic *Staphylococcus strains* is the ability to produce free coagulase and bound coagulase (clumping factor) [30]. While clumping factor activity is a defining characteristic of S. aureus, strains of coagulase-negative Staphylococci have also been reported to be positive in this reaction [31]. A high proportion of coagulase-negative *Staphylococcus strains* expressed surface proteins likely to produce positive reactions with the Staphytect test, which is more generally designed for the identification of S. aureus and to highlight the errors that could occur with such agglutination kits in line with the results of Personne et al. (1997) and Cuny et al. (1999) [32-33].

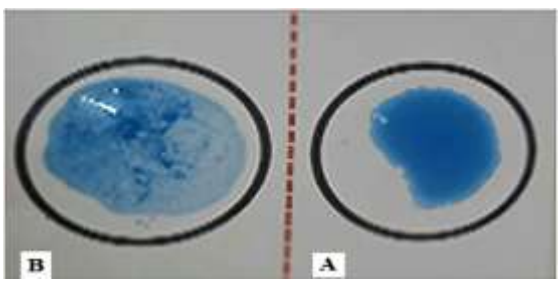

Figure 3. Staphytect Plus assay, A-Control Test, B-Positive Test.

Antibiotic susceptibility testing

The emergence of bacterial Resistance to antibiotics is a significant public health issue. A dual strategy is being implemented to combat this phenomenon: reducing antibiotic prescribing to limit selection pressure [34]. In the present study, 12 antibiotics commonly used in human medicine in Algeria were selected (Table 1).

Table 1. Resistance and sensitivity of *Staphylococcus strains* to different antibiotics

Tested antibiotics	Code	Disc load	Critical diameters (mm)			104100	6310111	6104100
			R	I	S			
Penicillin	PG	10 UI	≤28	-	≥29	R	R	R
Oxacillin	OX	1 µg	≤19	-	≥20	R	R	R
Erythromycin	E	15 µg	≤13	14-22	≥23	I	R	I
Trimethoprim + Sulfamethoxazole	SXT	1.25/23.75 µg	≤10	11-15	≥16	S	S	S
Tetracyclines	Te	30 µg	≤14	15-18	≥19	S	R	S
Vancomycin	Va	30 µg	-	-	≥15	S	R	S
Clindamycin	Cm	2 µg	<14	15-20	≥21	R	S	I
Ciprofloxacin	CIP	5ug	≤15	16-20	≥21	S	S	S
Cephalothin	KF	30ug	<14	15-17	>18	R	R	R
Nalidixic acid	NA	30ug	<13	14-18	>19	R	R	R
Gentamicin	GN	10ug	≤12	13-14	≥15	S	S	S
Chloramphénicol	C	30ug	≤12	13-14	≥18	S	S	S

The most remarkable result of this study is that the resistance to penicillin, oxacillin, cephalothin, and nalidixic acid was high, with a rate of 100%. In a study by Heath et al. (2023) [35], the incidence of *S. capitis* epidemics has often been associated with environmental sources. *S. capitis* isolates are frequently associated with SCCmec mobile genetic elements, a vector for exchanging resistance genes between staphylococcal species. The SCCmec cassette has been characterized as providing resistance to β-lactam antibiotics and demonstrating reduced susceptibility to vancomycin [36]. Furthermore, resistance to one antibiotic confers resistance to another, known as cross-resistance [37]. According to Carle (2009) [38], bacteria are considered multi-resistant when, due to an accumulation of natural and acquired resistances, they are no longer sensitive to more than a small number of antibiotics (Table 2). They are then resistant to several antibiotics or pharmacological classes of antibiotics. Methicillin-resistant *Staphylococcus capitis* (MRSC) has recently been described as an emerging cause of nosocomial bacteremia. This resistance could become a serious problem for the medical sector [39].

Table 2. Multiple antibiotic resistance among staphylococcal strains

Biotypes	Resistant strains	Number of resistant strains versus number of antibiotics						
		1	2	3	4	5	6	7
4 1 0 4 1 0 0	1					*		
6 3 1 0 1 1 1	1							*
0 0 0 4 1 0 1	1				*			

Despite their significant impact on human health, the pathogenicity and antibiotic resistance of *Staphylococcus warneri* is currently the subject of little research [40]. S. warneri isolates are generally resistant to beta-lactam antibiotics, with the mecA gene encoding resistance to most of them. However, some strains resist non-beta-lactam antibiotics, such as vancomycin [41]. Increased use of antibiotics has led to a rise in the incidence of multi-resistant staphylococci [42]. Resistance can also arise from genetic mutations that alter drug targets or enhance drug expulsion, as well as enzymatic degradation of antibiotics by specific bacteria [43].

Furthermore, biofilm formation provides a protective barrier, complicating treatment efforts and necessitating enhanced antibiotic stewardship and monitoring strategies [44].

Biofilm formation test

Our results showed that our strains were slim-producing, with black colonies and a dry crystalline consistency (Figure 4).

The organization of cells into biofilms compromises the ability of antimicrobials to penetrate bacterial cells, preventing the accumulation of antibiotic concentrations [45]. Biofilm-producing cells are resistant to antibiotics and the immune system, leading to the recurrence of infection [46]. The dye congo red interacts directly with specific polysaccharides, forming colored complexes so that slime-producing strains produce black colonies on congo red medium and non-producing strains red colonies [47]. Methicillin-resistant *S. capitis* has been reported to form a viscous biofilm on medical devices [48]. In addition, *S. warneri* can form a biofilm carrying more antibiotic-resistance genes than negative isolates [40]. Research by Qu et al. in (2020) [49] revealed that biofilm formation by *S. capitis* is significantly influenced by hyperosmotic conditions, such as high NaCl, KCl or $MgCl_2$ concentrations. These conditions stimulate the expression of the IcaAdBC gene cluster, essential for biofilm maturation; the study also highlights the importance of the physicochemical properties of biomaterial surfaces. Specifically, surfaces with high levels of oxidized carbon species are more conducive to biofilm formation. Indeed, they facilitate the immobilization of the extracellular polymeric matrix (EPS), mainly composed of polysaccharides [49]. The chemical composition of these effluents, rich in various antibiotic residues, acts as a selective pressure, favouring the survival of resistant strains of *S. capitis* [50].

Figure 4. Biofilm test.

CONCLUSIONS

Two findings about the risk of biofilm formation in antibiotic resistance were presented in this study. On the one hand, most isolates of *Staphylococcus* sp. produce different levels of biofilm, and they are all resistant to antibiotics at different levels. On the other hand, biofilm formation increases bacterial survival after exposure to antibiotics. This adds urgency to the additional investigations to examine the underlying resistance mechanisms and identify innovative strategies for preventing and eradicating biofilms, which would curtail the dissemination of resistant bacterial strains through effluents, and to formulate effective strategies to alleviate this escalating environmental and public health issue.

Conflict of interests

No conflict.

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