PERIODICUM BIOLOGORUM
VOL. 123, No 1–2, 19–27, 2021
DOI: 10.18054/pb.v123i1-2.6850

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original research article

Disinfectant as Removal Agent of the Pre-Formed Biofilm by *Staphylococcus* spp. Isolated from Dental Clinics in Taif, KSA

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Keywords: Biofilm, Dental clinics, Staphylococcal infection.

Abstract:

***Objective**: To identify staphylococcal microorganism isolated from dental clinics, detect the impact of some disinfectants on removing the preformed biofilms and to demonstrate the adhesion of cells on the surfaces of some materials used for manufacturing the dental material using Scanning electron microscope (SEM). **Material and methods**: Out of seventy totally different swabs, twelve staphylococcal isolates were recovered. All isolates were tested for susceptibility to thirteen antimicrobials. The isolates were screened for biofilm production using microtiter plate (MtP) test, moreover the efficacy of some disinfectant as removal the agents of preformed biofilm. Scanning electron microscope (SEM) was used for demonstrating biofilm formation by selected isolates on stainless-steel and rubber surfaces. **Results**: Of twelve staphylococci isolates, 33.3, 16.6 and 50 % were strong, moderate and weak biofilm producers, respectively. Adhesions of the preformed biofilm were found to be reduced within the presence of Betadine and sodium bicarbonate. SEM images showed the capability of biofilm formation by S. aureus H3 and S. hominis M0401 on stainless-steel and rubber surfaces. **Conclusion**: We conclude that establishing effective preventive ways for well-practiced infection management is crucial to prevent staphylococcal infections and promote a secure surroundings within the dental clinics by eradicating biofilms formed on medical devices. These substances may be betadine and sodium bicarbonate.*

INTRODUCTION

Healthcare associated infections (HAIs) referred to as nosocomial infections occur worldwide and have an effect on each developed and resource-poor countries. Infections acquired in health care settings are among the foremost causes of mortality and morbidity among hospitalized patients, visitor and staff (1). Approximately 70th of HAIs are caused by multidrug-resistant pathogens (MDRP), by the ability of the great majority of them to powerfully adhere to biotic (mucosal and soft tissues) or abiotic (medical devices and instruments) surfaces, and to grow in sessile mode to create a biofilm (2). Infection management is very stressed in today's practice of dentistry. Research has shown that improper disinfection of the dental environment will transmit infectious diseases and encourage be a health hazard to each dental personnel, as well as patients (3). Though it's well-known that the dental surroundings, which incorporates the instruments, dental materials, and dental units, can be means for cross-contamination, there's very little information on the microbic involvement. Transmission of diseases in a dental setting

can occur, (i) From the patient to the dental worker, (ii) from the dental worker to the patient, (iii) from one patient to the other, (4) from the dental office to the community(4-5). Regarding 60–70% of hospital infections are caused by medical devices contaminated with bacteria (6).

Staphylococci are the foremost microorganisms that cause the microbic infections associated with the biofilms, that has been formed on medical devices. this is due to the fact that staphylococci are normal commensal microflora on human skin and mucosal surfaces(7,8). Additionally, the survival of Staphylococci within the oral cavity depends on their successful adhesion to dental surfaces and their ability to develop into biofilms, called dental plaque (9).

Therefore, the main aims of this work: (i) isolation and molecular identification of the biofilm-producing staphylococcus strains collected from totally different dental clinics in taif, KSA; attending patients, staff workers and surfaces of medical devices and (ii) finding out the influence of some disinfectants on adhesiveness and biofilm formation, and (iii) demonstrating the attachment of biofilm forming Staphylococci on the surfaces of some materials used for manufacturing of the medical devices.

MATERIALS AND METHODS

Collection of specimens

A total of twenty swab specimens were randomly collected from completely different gingival sulcus in oral cavity of patients attending dental clinics in taif city, KSA. in addition, thirty samples were taken from hand (n=15) and nasal carriage (n=15) of clinic staff workers. Different samples were taken from varied inanimate surfaces within the clinical dental setting including dental chair units (n=10) and suction tips (n=10). All samples were placed directly in sterile screwed cap tubes with 5 ml nutrient broth, transferred to microbiology Laboratory at Department of Biology, Faculty of Science, Taif University and incubated aerobically at 37 °C for 24 h. each sample was sub-cultured on mannitol salt agar and nutrient agar plates. The resultant colonies with visually distinguishable morphology were selected and re-streaked on the same medium and incubated at 37 °C for 24-48 h. This step was repeated till getting the single pure cultures. The isolates were initial confirmed to the genus level by colony and cell morphology, catalase production, oxidase activity, motility and Gram reaction.

Sequencing of 16S rRNA genes

Partial sequence of 16S rDNA gene was amplified by polymerase chain reaction (PCR) using the genomic DNA as template and bacterial universal primers, 27F (5′-gagtttgatcactggctcag-3′) and 1492R (5′-tacggctacctt gttacgactt-3′).PCR products were purified using DNA-purification kits and send to Macrogen Co, Seoul, Korea for sequencing using 518F and 800R sequencing primers. The obtained sequences were compared for similarity with the reference species of bacterium obtainable in genebank database, using the NCBI/BLAST at http://www.ncbinlmnih.gov/. The nucleotide sequence information were deposited at the EMBL nucleotide sequence info and therefore the accession numbers were obtained.

Antibiotic susceptibility

The susceptibleness of the tested isolates to thirteen antibiotics as determined by standard Kirby Bauer's disc diffusion technique as suggested by the Clinical and Laboratory standard Institute (CLSI) guidelines (10).

Biofilm formation assay using microtiter plates (MTP) method

The biofilm formation assay in microtiter wells was performed as previously represented by Christensen et al. (11). Strains were classified into four classes consistent with the classification mentioned by Christensen et al. (11) with slight modification as diagrammatical by Stepanovic et al. (12). The interrupt absorbance (Ac) was the mean absorbance of the negative control. Strains were classified as follows: A= Ac = no biofilm producer (0) ; $Ac < A = (2 \times Ac)$ = weak biofilm producer (+) ; $(2 \times Ac) < A = (4 \times Ac)$ = moderate biofilm producer (++) ; $(4 \times Ac) < A$ = robust biofilm producer (+++). All tests were distributed in triplicate and also the results represented as mean values ± standard deviations (SD).

Efficiency of commercial disinfectants as removal agent of the pre-formed biofilm

Five commercial disinfectants were chosen according to their common use in Hospitals for cleansing, disinfection and sterilization of patient-care medical devises and personal hygiene. All disinfectants were purchased from Saudi market, Taif, KSA. the subsequent disinfectants were used: Betadine (0.1, 1, 2, and 5%), sodium bicarbonate (0.1, 2, 5, and 10%), Avohex Listerine and mouthwashes (1, 10, 20, and 100%), and ethyl alcohol at concentration (10, 30, 70, 90%), All disinfectants were diluted with sterile distilled water directly before use under sterilization condition. The results of disinfectants on removing preformed biofilm by biofilm forming isolates were determined using microtiter plates technique. Biofilm production was measured as previously represented within the biofilm production assay in the previous experiment. after incubation, the obtained bacterial culture on MTP wells were washed 3 times with three hundred µl of various concentrations of the tested disinfectants rather than phosphate buffered saline (PBS, pH = 7.4). Negative control wells contained 250 µl of un-inoculated BHIB while, positive control wells were contained inoculated BHIB. Both control wells were washed 3 times with PBS, (pH = 7.4) and also the tested disinfectants weren't applied. At-

tached bacteria were fixed with sodium acetate (2%), afterwards the wells were stained with crystal violet 1% for 5 min. The wells gently rinsed with tap water, air dried and also the absorbance (A) determined at 630 nm. Based on A values made by bacterial films, strains were classified into four classes as delineated before in biofilm formation assay.

Demonstrating attachment and biofilm formation by SEM

Biofilm formation was demonstrated using SEM to illustrate the attachment of cells to the stainless-steel and silicon rubber surfaces (suction tips) as common materials used for medical devices (13,14).

RESULTS

Isolation and molecular identification of bacteria

Twelve (17.4%) *Staphylococcus* spp. isolates that collected from samples from completely different dental clinics; attending patients, staff workers and surfaces of medical devices were selected consistent with their morphological and biochemical characteristics (data not shown). The incidence of staphylococcal isolates from different samples was illustrated in Table (1).

Table 1. *Incidence of* Staphylococcal *isolates in different samples.*

Samples/Swabs	Number of samples/swabs	Number (%) of recovered *Staphylococcal* isolates
Hand staff	15	**4 (26.6)**
Oral cavity (gingival sulcus)	20	**5 (20.0)**
Surfaces of dental chair unit	10	**1 (10.0)**
Surfaces of suction tips	10	**0 (0.0)**
Nasal carriage staff	15	**2 (13.3)**
Total	**70**	**12 (17.14)**

All isolates were subjected to additional identification by determinative the partial sequence of 16S rRNA gene (continuous stretches of approximately 700-1145 bp). The species were initially determined by the BLAST program on NCBI (http://www.ncbi.nlm. nih.gov/) supported the 16S rRNA sequences of type strains obtainable in NCBI database. The identity and coverage percentages were ranged from 99 -100% and 96 -100%, respectively (Table

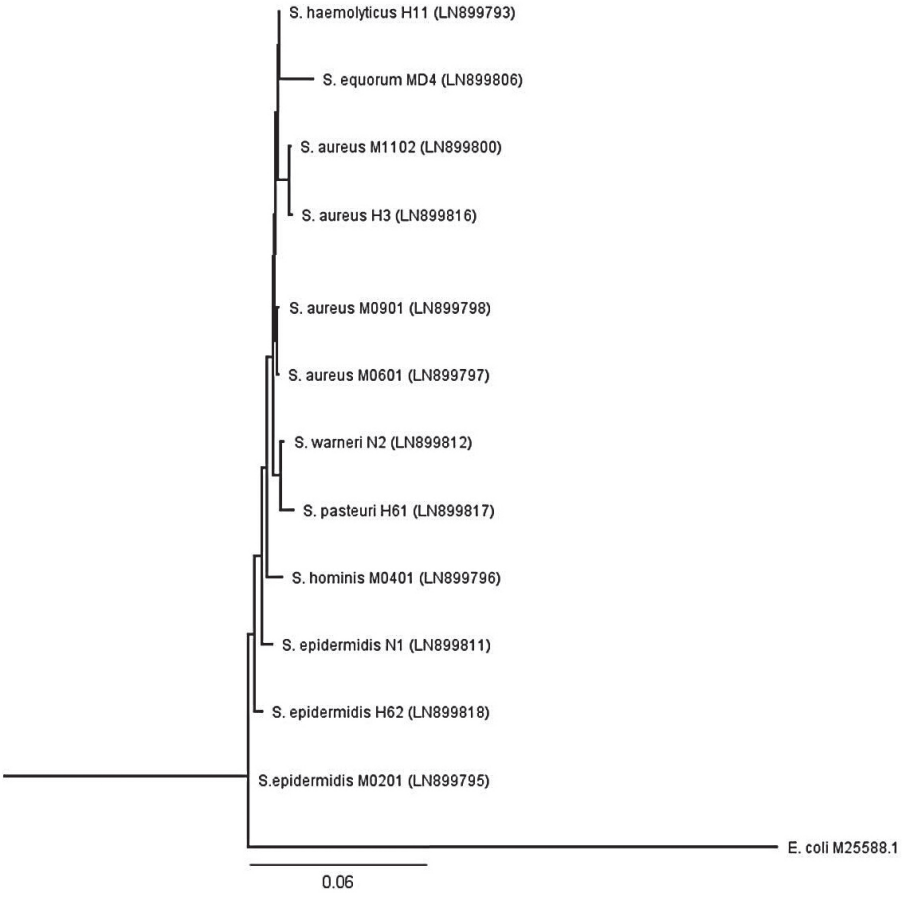

Figure 1. *Neighbor-joining phylogenetic tree based on 16S rRNA sequence analyses of the isolated bacterial strains using* Escherichia coli *(M25588.1) as out of group.*

Table 2. *Identification of* Staphylococcus *spp. according to partial sequencing of 16S rRNA and its accession numbers provided by EMBL Genebank.*

Source of Isolation	Nucleotide length (bp)	Blast results in NCBI database			Suggested Name	Accession No. at EMBL
		Accession No.	% identity	% coverage		
Hand staff	976	KF933780.1	99	98	*Staphylococcus aureus* H3	LN899816
Oral Cavity	909	JX866757.1	99	99	*Staphylococcus aureus* M0601	LN899797
Oral Cavity	950	KT339326.1	99	96	*Staphylococcus aureus* M0901	LN899798
Oral Cavity	986	KF933780.1	99	98	*Staphylococcus aureus* M1102	LN899800
Hand staff	914	KF056930.1	99	100	*Staphylococcus epidermidis* H62	LN899818
Oral Cavity	899	KP670187.1	100	100	*Staphylococcus epidermidis* M0201	LN899795
Nasal Cavity	946	KM225753.1	99	99	*Staphylococcus epidermidis* N1	LN899811
Surface of dental chair unit	950	KJ920933.1	99	99	*Staphylococcus equorum* MD4	LN899806
Hand staff	899	KJ623587.1	99	100	*Staphylococcus haemolyticus* H11	LN899793
Oral Cavity	961	KR085944.1	99	98	*Staphylococcushominis* M0401	LN899796
Hand staff	1145	KJ623586.1	100	100	*Staphylococcus pasteuri* H61	LN899817
Nasal Cavity	946	KR027924.1	99	99	*Staphylococcus warneri* N2	LN899812

2). Some isolates observed high identity percentages (99 or 100%) with over one species, however, the primary nearest species was chosen within the present study to determine a published name of the obtained isolates. The obtained partial 16S rRNA gene sequences were deposited within the European molecular biology Laboratory (EMBL) database. Identification of the isolated bacteria was confirmed and strains were given accession numbers. The suggested names and accession numbers of the isolated strains were given in Table 2.

According to the obtained results, four Staphylococcus; *S. aureus* H3, *S. pasteuri* H61, *S. epidermidis* H62, and *S. haemolyticus* H11 strains were isolated from hand staff. From the collected oral cavity swabs, five strains were isolated and known as *S. epidermidis* M0201, *S. hominis* M0401, *S. aureus* M0601, M0901 and M1102 (Table 2). in addition, *S. epidermidis* N1, *S. warneri* N2, were isolated from nasal carriage staff, whereas only *S. equorum* MD4 was isolated from surfaces of dental chair unit.

Interestingly, high similarity of the isolated strains at species level with none interference was determined when *E. coli* was used as out of group for constructing phylogenetic tree (Fig.1). Supported the partial sequence of 16S rRNA gene, no correlation between the source of isolation

Table 3: *Antibiotic resistant profile of Staphylococcus sp.*

Source of Isolation	**Identified isolates**	**Antibiotic susceptibility**													**% Resistance**
		TE	**VA**	**GN**	**AK**	**CIP**	**NOR**	**E**	**C**	**PB**	**AMC**	**OX**	**FOX**	**CAZ**	
Hand staff	*S. aureus* H3	R	S	S	S	S	S	S	S	R	R	R	S	R	**5**
Oral Cavity	*S. aureus* M0601	R	S	S	S	S	S	S	S	R	S	S	S	S	**2**
Oral Cavity	*S. aureus* M0901	S	S	S	S	S	S	R	S	R	S	S	S	R	**3**
Oral Cavity	*S. aureus* M1102	S	S	S	S	S	S	R	S	R	S	S	S	S	**2**
Hand staff	*S.epidermidis* H62	S	S	S	S	S	S	R	S	R	S	S	S	S	**1**
Oral Cavity	*S.epidermidis* M0201	S	S	S	S	S	S	S	S	S	S	S	S	S	**0**
Nasal Cavity	*S. epidermidis* N1	S	S	S	S	S	S	R	S	S	S	S	S	S	**1**
Medical devices	*S. equorum* MD4	S	S	S	R	S	S	R	S	S	S	S	S	S	**2**
Hand staff	*S.haemolyticus* H11	R	S	R	S	R	R	S	S	S	S	S	S	R	**5**
Oral Cavity	*S.hominis* M0401	R	S	S	S	S	S	R	S	S	S	S	S	S	**2**
Hand staff	*S.epidermidis* H62	S	S	S	S	S	S	R	S	S	S	S	S	S	**1**
Nasal Cavity	*S. warneri* N2	S	S	S	S	S	S	R	S	R	S	S	S	S	**2**
No. Of antibiotic resistant isolates from total of 12 isolates		4	0	1	1	1	1	7	0	6	1	1	0	3	
% antibiotic resistance		33.3	0	8.3	8.3	8.3	8.3	58.3	0	50	8.3	8.3	0	25	

TE, Tetracyclines; VA, Vancomycin; GN, Gentamicin; AK,Amikacin; CIP,Ciprofloxacin; NOR, Norfloxacin; E, Erythromycin; C, Chloramphenicol; PB,Polymyxin B; AMC,Amoxicillin-clavulanic acid; OX, Oxacillin; FOX, Cefoxitin; CAZ, Ceftazidime. (+) resistant; (-) sensitive.

and strain distribution within the phylogenetic tree was determined. As an example, *S. aureus* strain M1102 and H3, isolated from oral cavity and skin of 2 completely different persons, were enclosed within the same clad.

Antibiotic susceptibility

Most of *Staphylococcus* spp. strains obtained during this study failed to observe resistance profile for more than two antibiotics (Table 3). Out of twelve strains of staphylococci tested, 7 (58.3%) were resistant to erythromycin and six (50%) were resistant polymyxin B. Also, 4 strains (33.3%) were resistant to tetracycline. *S. aureus* H3 and *S. haemolyticus* H11 displayed resistant against five antibiotics, whereas *S. aureus* M0901 showed resistance to three antibiotics (Table 3). Herein, among listed 4 strains of *S. aureus*, just one showed resistance to oxacillin and it classified as methicillin-resistant *Staphylococcus aureus* (MRSA).

Interestingly, *S. epidermidis* M0201 isolated from oral cavity showed 100 percent susceptibility to all studied antibiotics. Moreover, S. pasteuri H61and each strains of *S. epidermidis* (N1 and H62) were showed 7.7 % antibiotic resistance. Whereas, *S. hominis* M0401, *S. warneri*

Table 4. *Quantitative detection of biofilm formation by* Staphylococcus *spp.*

Strain	Biofilm producing Category	A_{630nm}
S. aureus H3	+++	0.311±0.02
S.aureus M0601	+++	0.272±0.00
S.aureus M0901	+++	0.255±0.00
S.aureus M1102	+++	0.439±0.02
S.epidermidis H62	+	0.109±0.00
S.epidermidis M0201	+	0.110±0.01
S.epidermidis N1	+	0.077±0.00
S.equorum MD4	++	0.171±0.01
S.haemolyticus H11	+	0.122±0.01
S.hominis M0401	++	0.210±0.01
S.pasteuri H61	+	0.080±0.01
S.warneri N2	+	0.125±0.00

A_{630nm} absorption at 630 nm. (+) weak, (++) moderate, (+++) strong biofilm producer.

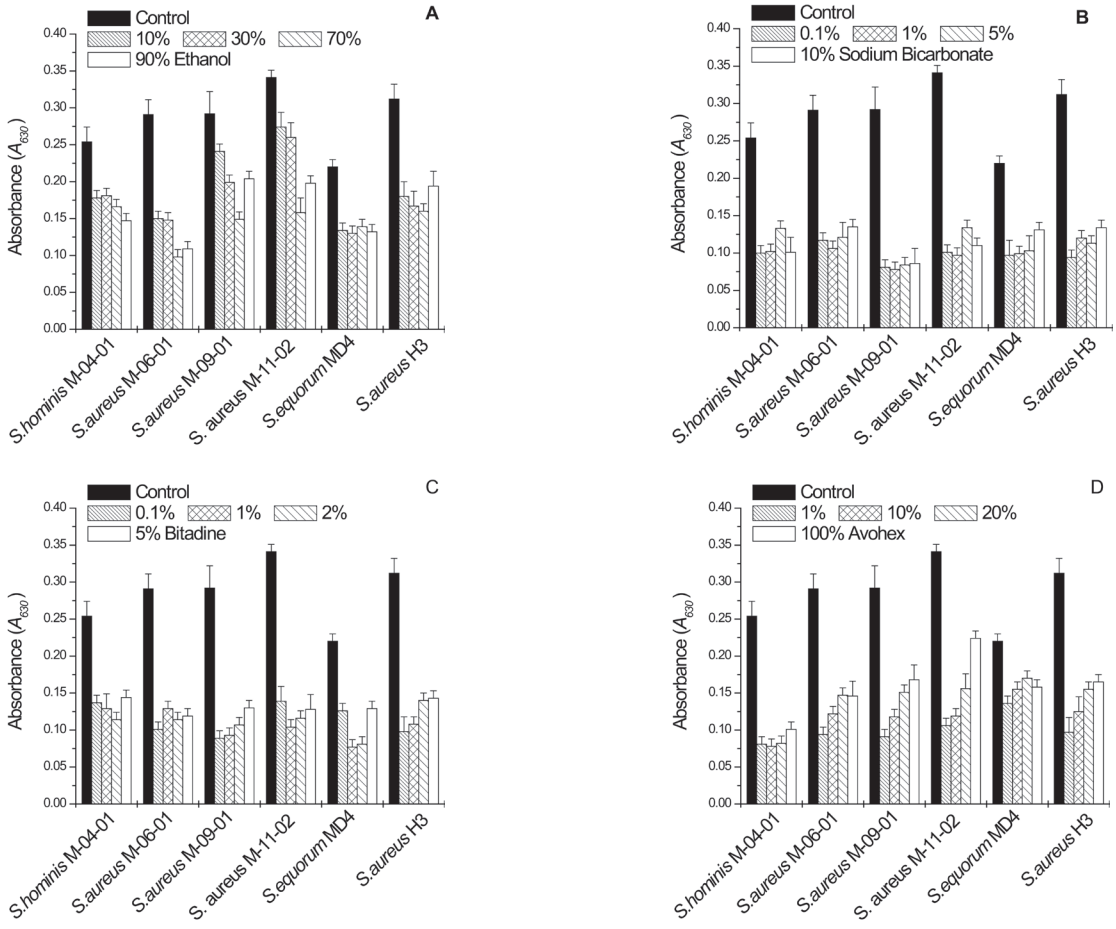

Fig. 2. *Effect of different disinfectants on biofilm formation by* Staphylococcus *spp. (A): Ethanol (B): Sodium Bicarbonate (C): Betadine (D): Avohex mouthwash*

N2 and *S. aureus* (M0601 and M1102) were showed 15.4 % resistance. On the opposite hand, cefoxitin, antibiotic and vancomycin could also be effective antimicrobial agents against the tested isolates.

Biofilm forming ability of Staphylococcus spp.

All tested isolated showed ability to form biofilm on polystyrene surface in several patterns (Table 3). *S. aureus* strains M0601, M0901, M1102 and H3 were strong biofilm producers (+++), whereas *S. hominis* M0401 and *S. equorum* MD4 had moderate ability to form biofilm (++). The other staphylococcus strains were weak biofilm producers (+). Concerning 50% staphylococcal isolates were biofilms producers (strong and moderate).

Effect of disinfectants on pre-formed biofilm

The result of various commercial disinfectants on removing the preformed biofilm of the moderate (++) and strong (+++) biofilm forming strains was studied. Four completely different disinfectants enclosed ethanol, sodium bicarbonate, betadine, and also the mouthwash avohex were examined (Fig.2).

Most strains were affected by washing with ethanol. *S. aureus* M0601 was the foremost affected strains, wherever its ability to form biolfilm modified from strong (+++) to weak (+) class by washing obtained culture with ethanol (70%). However, biofilm adherence of *S. hominis* M0401 was unaffected by washing with ethyl alcohol concentration up to 90%. *S. equorum* MD4 became weak biofilm producer once 10th ethanol was used in washing buffer (Fig. 2A). Generally, it absolutely was detected that washing of culture media with ethanol inhibited biofilm adherence by the tested staphylococci and this inhibition was directly proportional to ethanol concentrations. On the opposite hand, tested bacteria *S. equorum* MD4 and both *S. aureus* (M0601 and H3) were additional sensitive to ethanol particularly 70th concentration. In addition, biofilm adherence varied consistent with wash cultured media with different sodium bicarbonate concentrations (Fig. 2B). Generally, bicarbonate was ready to drastically reduce the adherence of bacterial biofilm. As shown in (Fig. 2C), washing with betadine inhibited biofilm adhesion by the tested staphylococci. The result of various concentrations

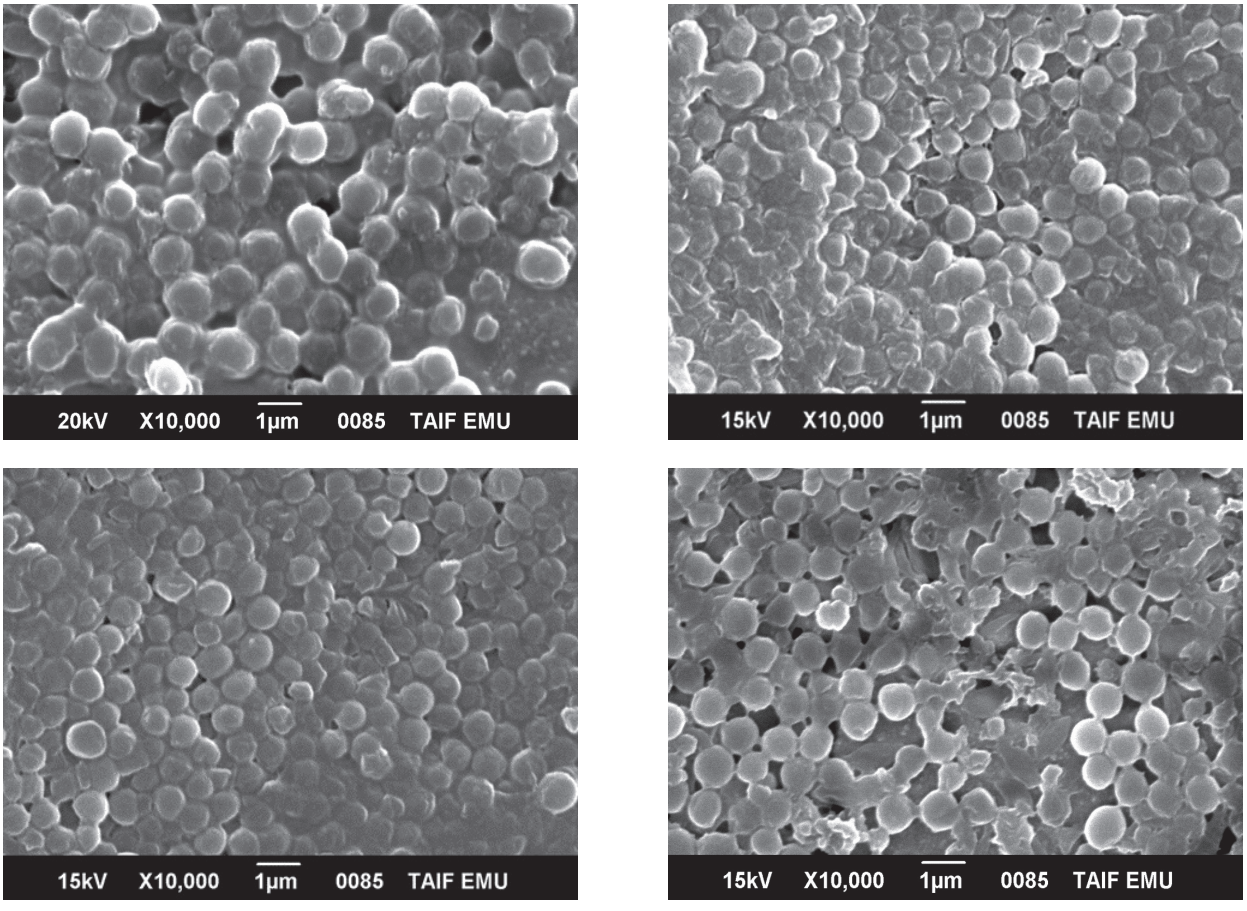

Figure 3. *Scanning electron microscope of* S. aureus *H3 cells adhered on stainless steel (A) and suction tip surface (B),* S. hominis *M0401 cells adhered on stainless steel (C) and suction tip surface (D). Black arrows indicated cells impeded inextracellular secretions, White arrows indicated extracellular matrix between cells.*

of Avohex mouthwash on biofilm formation by *Staphylococcus* spp. was studied (Fig. 2D). The greatest result of Avohex against all strains was obtained at 1% concentration. *S. hominis* M-04-01 was the foremost affected strain, where it was modified from strong to weak biofilm producer once (1%) Avohex was applied, while *S. equorum* MD4 was additional resistant to all Avohex concentrations. Moreover, Avohex mouthwash at 20% and 100% concentrations shows lower activity against four *S. aureus* strains (Fig.2D). In general, the lowest examined concentration (1%) of Avohex showed additional disinfection result compared with the highest (100%) concentration. For all staphylococcus spp., biofilm formation was affected at the 1% and 10% Listerine mouthwash concentrations. *S. aureus* (M0901 and M1102) exhibited slightly resistant to Listerine at 20% and 1000% concentrations. However, *S. hominis* M0401, *S. equorum* MD4 and *S. aureus* (M0601 and H3) were the foremost affected strains at full concentrations (Fig 1E).

Demonstration of biofilm formation on medical device surfaces

Scanning electron microscope (SEM) is that the commonest methodology for examination and characterization of biofilm development on medical devices. *S. aureus* H3, and *S. hominis* M0401 were subjected for SEM to Illustrate the adhesion of cells to stainless-steel and suction tips (rubber) surfaces after 8 days incubation at 37 °C (Fig.3). SEM photos showed that, the staphylococcus cells seemed to be impeded in extracellular secretions. Moreover, extracellular matrix between cells could be additionally determined.

DISCUSSION

Although the contamination of the dental surroundings and personnel through aerosol contamination could be a definite source of cross contamination; there's very little information on the microbic involvement of the dental environment. In our study, the frequency of isolates of staphylococcal varied in numerous sample sources. Comparable results showing variations within the incidence of staphylococcal isolates among clinical and environmental samples have been reported (15-18). These variations could also be because of environmental factors, nutritional requirements, or virulence factors.

A high rate of resistance to erythromycin (58.3%) and polymyxin B (50 %), however all were susceptible to vancomycin, chloramphenicol and cefoxitin. These results support the finding by Ito et al. (19) and Wojtyczka et al. (20).Generally, high to moderate prevalence of antibiotic resistance may be due to their biofilm forming nature. Consequently, investigations to understand the pathogenesis of those infections have targeted upon the method of adherence of those microorganisms on the collected samples. Their high prevalence of antibiotic resistance could be due to their biofilm forming nature. In our study, regarding 50th staphylococcal isolates were biofilms producers (strong and moderate), that is entirely according to previously reported data (21-24).

In ordering to check the activity of ethanol as removable of pre-formed biofilm, it was determined that the adherence of tested biofilm manufacturing bacteria was reduced and such reduction was concentration dependent. Peters et al. (25) has reported similar findings that mentioned that ethanol is effective at eradicating biofilm-embedded bacteria.

Sodium bicarbonate was found to be able to drastically reduce the adherence of bacterial pre-formed biofilm. Several studies have shown that sodium bicarbonate at high concentrations up to has an antimicrobial effect over many microorganisms isolated from the oral cavity (25-27). Pratten et al. (28) reported that bicarbonate is able to disrupt mature dental plaque grown in vitro. Moreover, increasing of betadine concentrations repressed biofilm adhesion by our isolates. Betadine contains povidone-iodine, because the active ingredient, that more effective in reducing biofilm formation. Povidone-iodine (PI) could be a complex of polyvinyl pyrrilidine and triiodine ions that's broadly applied as an antiseptic in several surgery.[29]Generally, reduction and disrupting of preformed biofilm using betadine, were concentration and isolate dependent (30-31).

The lowest concentration (1%) of tested Avohex mouthwash was more effective as removing pre-formed biofilm as compared with the highest (100%) concentration. A possible explanation could be that the principal ingredient of Avohex® is 0.2% chlorhexidine gluconate. This chemical antimicrobial that kills each Gram positive and Gram negative microbes attacked the bacterial cytoplasm or inner membrane. it's been used widely in hand hygiene, skin disinfection and mouth rinse within the prevention and treatment of periodontal diseases and dental caries (32,33).

Essential oil-containing mouthwash like Listerine showed activity against some tested bacterial strains, however not others. Essential oil such as Eucalyptol, Menthol, Methyl salicylate and thymol killed microorganisms by disrupting their cell walls and by inhibiting their enzyme activity. It can prevent bacteria from aggregating with Gram positive species and extract endotoxins from Gram negative pathogens (34).

Discussing the results of biofilm formation by *S. aureus* H3 and *S. hominis* M0401 on stainless steel and rubber using Scanning electron microscope (SEM) conclude that the extracellular matrix between the tested cells ar most likely exopolysaccharides, that responsible primarily for cell adhesion to each other and to their surrounding surfaces (35). Moreover, obtained result's harmonious with other studies that refer rubber and stainless

steel as being particularly disposed to being colonized by *S. aureus* and *S. hominis* (36,37). A possible clarification may be that the adhesion of bacterial cells depends on several factors such as physiology and cell morphology and physico-chemical properties of the contact surface. Moreover, the ability of microorganisms to attach to abiotic substrate could referred to hydrophobic interactions and extracellular compounds (37).

Finally, the current study shed light on the harmful of contamination of an orthodontic clinical environment with biofilm forming staphylococcus spp. The commercial disinfectants couldn't completely take away the preformed biofilms developed by staphylococcus spp. The biofilm forming staphylococcus spp. such as *S. aureus* H3 and *S. hominis* M0401 have high ability to adhere to totally different surfaces like stainless steel and rubber. Although, the tested isolates were relatively susceptible to Cefoxitin, chloramphenicol and vancomycin, control of contaminations using antimicrobial drug may cause serious medical problems. This adds urgency to the search for new infection fighting compounds to regulate microbial infections by eradicating biofilms formed on medical devices. These substances may be betadine and sodium bicarbonate.

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