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Genetic Study of *S.aureus* that Resistance to Antibiotics and Isolate from Dental Plaque in Periodontitis Patients

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Abstract—Background*: Staphylococcus aureus is a significant human pathogen responsible for many illnesses. In the past 25 years, the prevalence of both community-acquired and hospital-acquired S. aureus infections has risen (1). S. aureus is a significant human pathogen in hospitals and communities, serving as a key cause of several illnesses. S. aureus colonies patients' teeth in the form of biofilm. Biofilms are the predominant form of bacterial proliferation in natural environments and are significant in clinical and dental illnesses. The most prevalent dental diseases include gingivitis, periodontitis, and dental caries, which are caused by microorganisms that constitute the normal flora in the form of biofilm. Biofilm is defined as a complex accumulation of microorganisms enclosed in a protective and adhesive matrix of polymeric substances, adhering to both inert and living surfaces.* ***Methodology****: In the current study, 50 samples (Swabs) were collected from patients suffering from gingivitis who attended Al-Kafeel Dental Clinics in the city of Najaf for the period from February 2024 to April 2024. The samples were transferred to the laboratory for the purpose of isolating and diagnosing bacterial species on culture media and knowing their resistance pattern to a number of antibiotics that are prescribed by dentists. 21 Specimens isolated were S.aureus, PCR was also used to identify detection (CTX-M) and (SHV) genes of S.aureus that encode resistance to antibiotics, in addition to testing the ability of bacteria to produce and form biofilm using ELISA technique.* ***Aim of study****: Detection (CTX-M) and (SHV) genes that code to ability of S.aureus to resist antibiotics its ability to biofilm formation.*

I. INTRODUCTION

Dental plaque constitutes a complex biofilm that develops on the hard tissues, specifically the teeth, in the oral cavity. Over 500 bacterial species exist in plaque, with colonisation occurring systematically. This process begins with primary colonisers adhering to the enamel salivary pellicle, followed by secondary colonisation through inter-bacterial adhesion. A variety of adhesions and molecular interactions enable these adhesive contacts, leading to plaque formation and, ultimately, to conditions such as dental caries and periodontal disease (2). Periodontal diseases affect the supporting structures of teeth. Gingivitis and periodontitis are common inflammatory diseases resulting from the polymicrobial biofilm (dental plaque) that forms on teeth in the absence of regular oral hygiene practices. Gingivitis is defined as a reversible inflammatory response that does not result in the loss of bone support, while periodontitis involves the destruction of the periodontal attachment and the alveolar bone (3). Periodontitis arises from a complex interaction between the microorganisms present in the dental biofilm and the host organism. The involvement of particular microorganisms and their byproducts in the onset and progression of disease remains ambiguous (4). The severity of periodontal disease is influenced by environmental factors, such as smoking, as well as host risk factors, including genetic susceptibility. Periodontitis results from microorganisms that attach to and proliferate on tooth surfaces, coupled with an overestimated immune response to these microorganisms. Studies investigating the causative pathogens of periodontal diseases in Korean patients indicate that S. aureus exacerbates dental diseases by forming biofilms with other pathogens linked to periodontal illnesses [5].

S. aureus is a significant sources of numerous human diseases. While it typically presents mild symptoms in healthy skin or soft tissue, it becomes a primary source of infections in certain environments, such as hospitals, accounting for over 80% of pyogenic diseases. S. aureus is linked to dentoalveolar infections and oral mucosal lesions in the oral tract. Staphylococcal colonisation has been observed in the tongue, saliva, mucosal surfaces, supragingival tooth surfaces, and the periodontal pocket [6,7]. The factors that increase the virulence of S. aureus associated with infections and diseases can be classified into surface-associated factors, degradative enzymes, and superantigenic toxins. The diversity and variability of these genes may affect the progression of an infection [8]. Staphylococcus aureus is increasingly recognised as a causative agent of severe invasive diseases such as osteomyelitis, septic arthritis, and pneumonia with empyema. The incidence of multi-drug resistant S. aureus has increased in both community and healthcare environments in recent decades [9]. The close closeness of bacterial cells in a biofilm enables cooperative metabolic activities, encourages horizontal gene transfer, and improves cell-to-cell communication [10]. Biofilms are generally associated with wet or humid surfaces, such as implanted medical devices and tubing in healthcare apparatus. Certain bacterial species, such as Staphylococcus aureus, may persist in a desiccated state for extended periods and are capable of building biofilms on dry clinical surfaces [11].

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The biofilm formation abilities of staphylococci, especially Staphylococcus aureus, have been thoroughly documented. Biofilm formation is essential for the survival and virulence of clinical staphylococci [8]. Different forms of S. aureus infections are associated with the formation of bacterial biofilms on native tissues or implanted biomaterials. The synthesis of polysaccharide intercellular adhesion (PIA) by enzymes encoded by the ica operon is the most thoroughly elucidated mechanism of biofilm formation in staphylococci [11]. Beta-lactam antibiotics are frequently employed in the treatment of bacterial infections. The rising utilisation of these agents is associated with the rise of beta-lactamase-mediated bacterial resistance and the ensuing proliferation of extended-spectrum beta-lactamase (ESBL) generating bacteria.[12] The selection pressure exerted by third-generation cephalosporins, especially in critical care environments, leads to amino acid changes facilitated by plasmid-encoded beta-lactamases. Amino acid changes result in the production of ESBLs capable of inactivating oxyimino-beta-lactam antibiotics [13]. Both of them Gram-positive and Gram-negative organisms produce beta-lactamases; however, this characteristic is more common in Gram-negative bacteria. Gram-positive organisms secrete beta-lactamase into the extracellular environment, whereas Gram-negative species release it into the periplasmic region.[14].

II. METHODOLOGY

2.1 Sample collection

50 samples were isolated from patients who attended Al-Kafeel dental teaching clinics (35 men and 15 women) for the period from February 2024 to April 2024, where swabs were taken from two types of dental plaque (34 supra-gingival plaque and 16 were a mix supra and sub-gingival plaque), Figure (1).

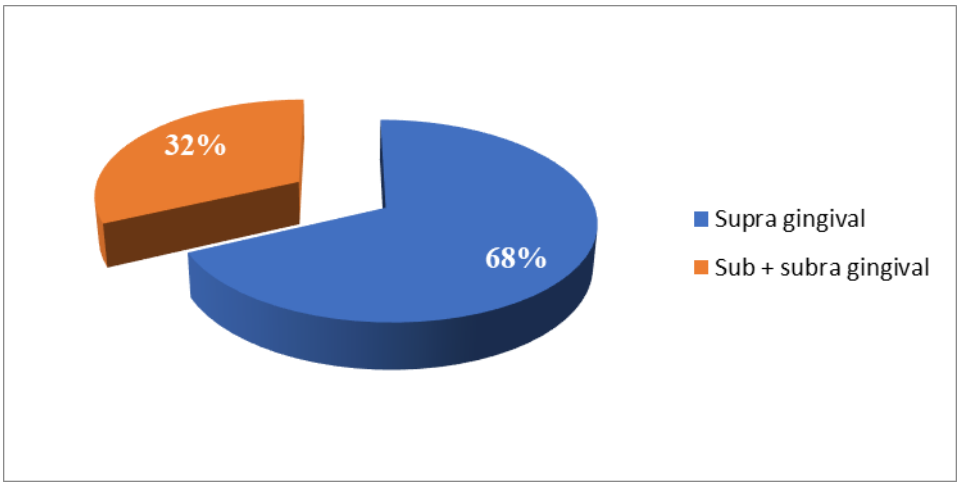

Figure (1): distrbution of swabs taken from pateints with periodentitis

2.2 Bacterial culture and identification

Dental plaque swabs were cultured to identify aerobic bacterial growth by inoculating in various culture media, including MacConkey's agar, Blood agar, Nutrient agar, and Mannitol salt agar (Oxoid/England), followed by incubation at 37 °C for 24-48 hours under aerobic conditions. Growth was assessed on the plates following 24 hours of incubation [15]. The Vitek 2 System, along with Gram-positive (GP) and Gram-negative (GN) colourimetric cards from BioMérieux (France), was employed to confirm the diagnosis of bacterial isolates.

2.3 Antibiotic Susceptibility

The antibiotic sensitivity test was conducted using the Kirby-Bauer method established in 1984 by Claus and Berkeley. Five millilitres of brain heart infusion broth media were dispensed into plain tubes and inoculated with a full loop of bacterial isolate. The inoculated tubes were then incubated at 37ºC for 24 hours. Subsequently, the inoculated broth was compared to the turbidity standard, and the density of the test suspension was adjusted to match a 0.5 McFarland tube. Bacteria were transferred using a sterile cotton swab streaked across the surface of a Mueller-Hinton agar plate (Difco-USA). The inoculated plates were allowed to dry for approximately one minute at room temperature. Selected antibiotic discs, including Amoxicillin/Clavulanic, Cefixime, Gentamicin, and Metronidazole, were placed on the bacterial plates using sterile forceps and incubated at 37ºC for 24 hours in an inverted position. Following overnight incubation of the inoculated plates, the diameters of the inhibition zones were measured in millimetres using a ruler. The inhibition zone results were determined based on the Clinical and Laboratory Standards Institute (15).

2.4 DNA extraction and PCR amplification

The genomic DNA of S. aureus was extracted using the boiling method described by Shakibaie et al. [16]. The concentration and purity of the extracted DNA were assessed using a nanodrop system. The identification of β-lactamase genes, bla CTX and bla SHV, in the positive ESBL isolates was achieved through PCR with specific oligonucleotide primers, as detailed in Tables 1 and 2.

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TABLE (1): Primers used in PCR amplification

Bla genes	Primer sequences	References
CTX-M (F)	ATGTGCAGTACCAGTAA	Perez, et al. [33]
CTX-M (R)	CCGCTGCCGGTTTTATC	
SHV (F)	CTTTACTCGCCTTTATCG	
SHV (R)	TCCCGCGGATAAATCACCA	

TABLE (2): Components of PCR

No	Components	Volume (µl)
1	PCR Master mix	12.5
2	Distilled water DNA-free	8.5
3	Forward primer (10 pmol/µl)	1.0
4	Reverse primer (10pmol/µl)	1.0
5	DNA template	2.0
	Total	25

The cycle parameters comprised an initial denaturation at 94°C for 3 minutes, subsequent template denaturation at 94°C for 30 seconds, annealing at 48°C for CTX and 60°C for SHV for 30 seconds, and extension at 72°C for 1 minute. The procedure was executed for 30 cycles, culminating in a final extension at 72°C for 10 minutes. The study utilised a positive control supplied by DVRC. Amplicons were subjected to electrophoresis on a 1.5% agarose gel and visualised post-staining with Red Safe under a transilluminator equipped with a digital camera (Biometra, Goettingen, Germany). A 100 bp ladder (KAPA, Germany) served as the molecular weight marker.

2.5 Biofilm formation test

his study utilised TCPM (Tissue Culture Plate Method) as the gold standard for detecting biofilm formation (17,18). A loopful of freshly cultured isolates was inoculated into 10 ml of trypticase soy broth containing 1% glucose. The inoculated broth was incubated at 37°C for 24 hours. Bacterial suspensions underwent a subsequent dilution of 1:100 using fresh medium. Separate wells of a sterile polystyrene tissue culture plate, consisting of 96 flat-bottom wells, were filled with 200 µL of the prepared bacterial suspension. Control organisms were similarly placed in the tissue culture plate. Furthermore, sterile broth was exclusively utilised to maintain sterility and to detect non-specific binding. Following a 24-hour incubation at 37°C, the plate was gently tapped to expel the well contents, which was subsequently washed with 200 µL of phosphate-buffered saline. The washing procedure was conducted four times to eliminate any residual bacteria in the wells. Sodium acetate (2%) was added to the wells and incubated for 30 minutes to fix the biofilms formed by bacteria adhered to the wells. Biofilms were stained with crystal violet at a concentration of 0.1%. Following a 30-minute period, the wells were rinsed with deionised water to eliminate any residual stain. Following drying, a micro-ELISA reader operating at a wavelength of 570 nm was utilized to measure the optical densities (OD) of stained bacterial biofilms. The experiment was performed in triplicate, and the mean of the three optical density (OD) values was determined. Optical density measurements demonstrated bacterial adhesion to the wells and biofilm development. The optical density values were assessed, and biofilm generation was categorised as strong, moderate, or non/weak (Table 3), in accordance with the technique used in previous studies (19,20).

TABLE (3): Bacterial biofilm test due to the TCP method

Optical densities value	Adherence	Biofilm formation
< 0.120	Non	None / Weak
0.120- 0.240	Moderate	Moderate
> 0.240	Strong	Strong

III. RESULTS

3.1 Bacterial Identification

In this study, 50 swabs were collected from people suffering from periodontitis and tooth caries, and the bacterial were obtained, which were identified as Gram-positive and gram negative bacteria using the Vitek system. The results in Figure (2) showed the dominance of S. aureus, which recorded the highest rate 21/50 (42%) of the total bacteria isolated from tooth caries, followed by S. mutans 13/50 (26 %), then by Lactobacilli (8/50)16%. While K .pneumoniae recorded (4/50) 8% isolation rate.

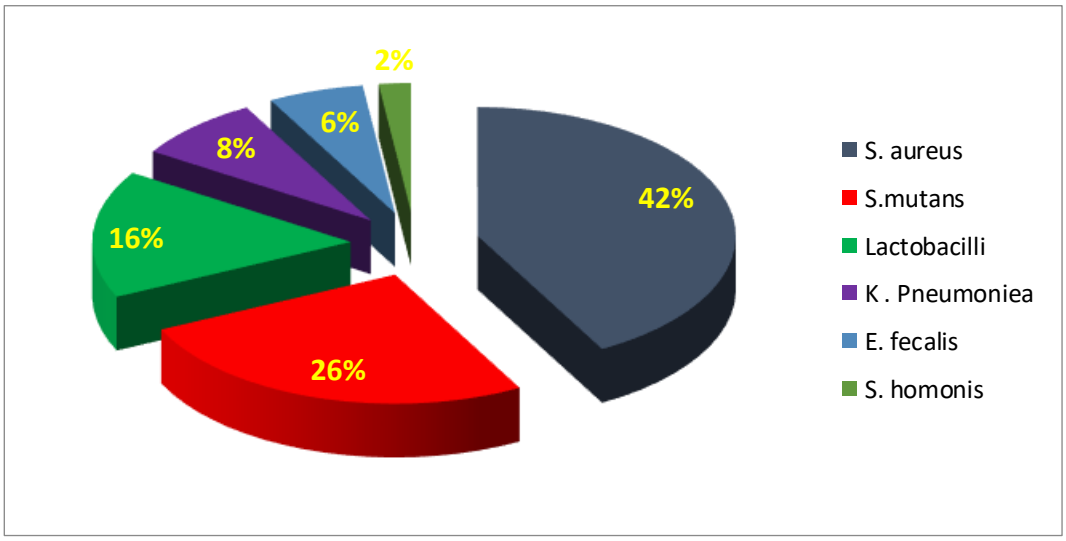

Figure (2): Percentage of bacteria species isolates

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3.2 Antibiotic Susceptibility

A drug sensitivity test was conducted for all S. aureus isolates involved in the study, which are implicated in dental plaque, against a group of antibiotics (Beta lactam). Resistance was assessed by measuring the diameter of the growth inhibition zone surrounding the antibiotic discs and comparing the results with those reported by [22]. The results indicated that S. aureus exhibited 100% resistance to Gentamicin and Metronidazole, while resistance to Amoxicillin and Cefixime was observed at 95%., Figure (3) Table (4):

TABLE (4): S.aureus resistance to beta lactam drug

No of Isolate	CFX	AMC	GN	MTZ
6	10	R	R	R
10	R	R	R	R
14	R	11	R	R
15	R	R	R	R
21	R	R	R	R
22	R	R	R	R
23	R	R	R	R
24	R	R	R	R
31	R	R	R	R
32	R	R	R	R
33	R	R	R	R
34	R	R	R	R
35	R	R	R	R
36	R	R	R	R
38	R	R	R	R
39	R	R	R	R
40	R	R	R	R
41	R	R	R	R
42	R	R	R	R
46	R	R	R	R
48	R	R	R	R

3.3 Detection of CTX, SHV genes and PCR amplification

The results of the present study showed that18/21 (85. 71%) isolates of S.aureus were positive result for CTX gene while 15/21(71.42%) were positive *SHV* genes, Figure (4 and 5).

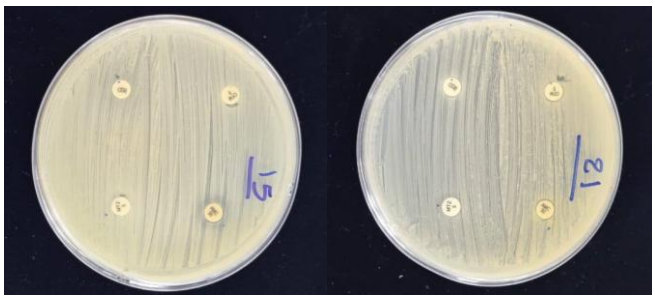

Figure (3): S.aureus to antibiotics on Muller Hinton agar

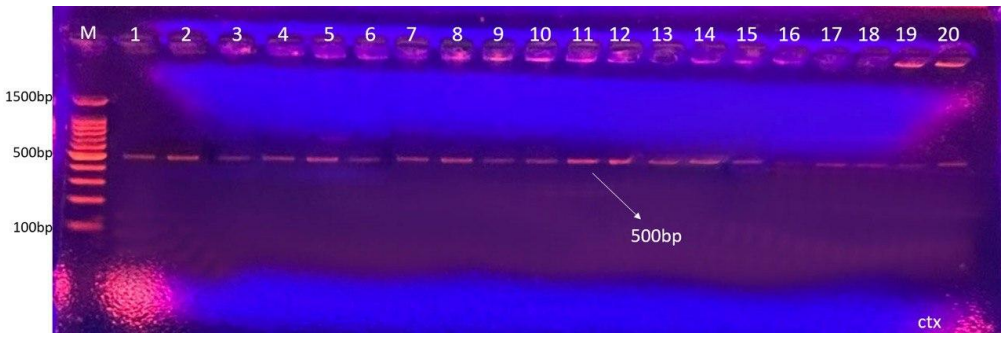

Figure (4): Gel electrophoresis for amplification of *CTX* gene by using convential PCR technique to demonstrate strains of S.aureus , M(marker or ladder), isolate (1-20 except 16 and 19 were positive at 500 bp) electrophoresis was performed at 70 volt for 80 min.

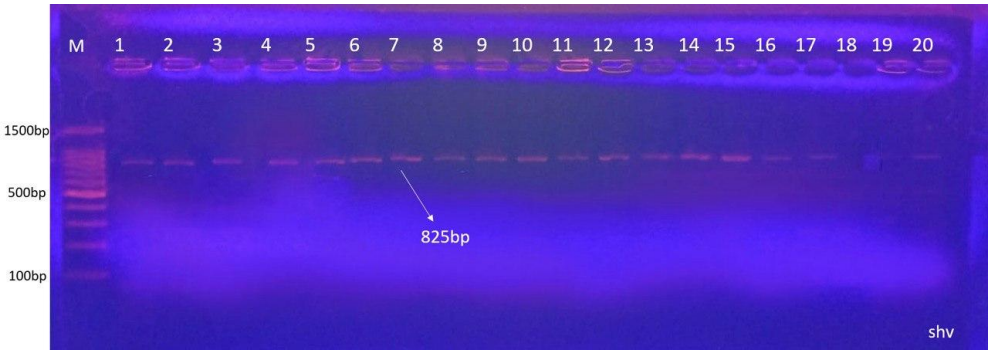

Figure (5): Gel electrophoresis for amplification of *SHV* gene by using convential PCR technique to demonstrate strains of S.aureus , M(marker or ladder), isolate (1-15 were positive at 825bp) electrophoresis was performed at 70 volt for 80 min.

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3.4 Detection formation of biofilm

The results of current study showed that 12/21(57%) of S.aureus were weak to produce biofilm (< 0.120) and 5/21 (24%) of isolates were strong to produce biofilm (> 0.240) while only 4/21 isolates (19%) were moderate (0.120- 0.240) , Table (5):

TABLE (5): biofilm formation test of S.aureus where ; W : weak (< 0.120) , M : Moderate (0.120- 0.240) and S : strong (> 0.240).

No of isolate	Biofilm production	Test Results
6	W	0.099
10	M	0.2148
14	W	0.0456
15	M	0.1229
21	W	0.0648
22	M	0.151
23	W	0.0948
24	W	0.0803
31	W	0.043
32	W	0.0542
33	W	0.0775
34	W	0.0697
35	W	0.093
36	M	0.1479
38	S	0.8406
39	W	0.1016
40	S	0.8013
41	S	0.6465
42	W	0.0552
46	S	0.7141
48	S	0.3537

IV. Discussion

Dental infections primarily result from various oral microorganisms, influenced by antibiotic resistance and biofilm formation [(21)]. Staphylococcus aureus is an opportunistic pathogen, with approximately 30-50% of healthy children and adults identified as carriers. It is a primary cause of S. aureus bacteremia (SAB) and other bloodstream infections.[22]

The mechanisms by which biofilms exhibit enhanced resistance to antibiotics remain inadequately elucidated. The proximity of bacteria within a biofilm to the matrix may impede the penetration of antibiotics through the exo-polysaccharide matrix. Biofilm bacteria demonstrate a reduced metabolic rate and infrequent division, leading to susceptibility to antibiotics that target cell wall synthesis, such as penicillins. Biofilms are associated with infections in specific human diseases [(22)]. Oral biofilm comprises bacterial cells, salivary polymers, and bacterial extracellular products that adhere to teeth. Accumulations of microorganisms alter the teeth and gingival tissues, leading to elevated concentrations of bacterial metabolites that result in dental disease. Dental plaque biofilms contribute to various dental diseases.

The high prevalence of multidrug-resistant bacteria poses significant implications for public health, particularly concerning healthcare delivery. Infections caused by these superbugs are linked to restricted treatment alternatives, significant healthcare expenses, and extended hospitalisations (23). Multidrug-resistant pathogens present therapeutic challenges and possess a significant potential for acquiring further resistance, leading to widespread dissemination within hospitals, thereby increasing the threat to infection control [24]. These organisms are linked to bloodstream infections, hospital-associated pneumonia, surgical site infections, and other nosocomial infections, contributing to increased admissions to intensive care units (ICUs), as well as elevated morbidity and mortality rates[25]. This variation may be ascribed to environmental factors, geographical location, educational attainment, and patients who received antibiotics prior to sample collection [26,27]. Factors including hormonal changes, depression, menopause, hospital-acquired infections, immunocompromised patients, and prolonged hospital stays contribute to an elevated risk of infections [28,29]. Antibiotic resistance genes represent a critical factor [30].

The CTX and SHV genes were the most frequently identified resistant genes in this study, present in the majority of isolates. A recent study conducted in North-eastern Nigeria identified the SHV gene as the most prevalent. The variation in the distribution of ESBL-resistant genes across geographical regions underscores the necessity for regular surveillance to inform antimicrobial therapy selection (31).

Plasmid-mediated ESBL genes present a significant risk for the horizontal transfer of resistance genes among bacterial populations. Outbreaks of ESBL-producing organisms present significant challenges for containment due to the limited therapeutic options available in many developing nations. Poor practices in infection prevention and control (IPC), coupled with inadequate antibiotic stewardship in many health institutions, may facilitate the emergence and dissemination of resistant bacteria worldwide.[32]

V. Conclusion

This study demonstrated the presence of two types of genes that cause drug resistance to treatments commonly used in dental clinics in most isolates of Staphylococcus aureus bacteria, in addition to the presence of diversity among the strains of this bacteria regarding the ability to produce biofilm.

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