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ISOLATION AND ASSESSMENT OF BIOFILM FORMING MICROORGANISMS ASSOCIATED WITH TOOTH DECAY IN KEFFI, CENTRAL NIGERIA

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ABSTRACT

Dental biofilm forming organisms are the major sources of oral diseases globally. The purpose of this study is to isolate and identify common microorganisms associated with tooth decay and assessing their biofilm forming potentials in a tertiary healthcare facility in Central Nigeria. Eleven (11) swabs samples were collected from the oral cavity of participants presented with tooth decay and were inoculated in Nutrient broth for 24 hours. The broth cultures were later standardized and inoculated on Mueller-Hinton agar and Sabouroud dextrose agar for the isolation of bacterial and fungal species respectively. The isolates were identified and characterized using standard microbiological techniques. Sixteen of the isolates were selected for quantitative biofilm formation assay which was performed by the microtiter plate and spectrophotometer assay. Among the microorganisms isolated from the oral cavity, *Streptococcus mutans* and *Lactobacillus acidophilus* had the highest percentage of occurrence (18.75%) while *Bacillus subtilis* and *Candida albicans* had the lowest percentage of occurrence (6.25%). Of the 16 isolates tested, 5(31.25%) were strong, 9(56.25%) were moderate and 2(12.5%) were weak biofilm formers. The absorbance was considered as an index of biofilm formation. This study has reported the predominance of bacterial species associated with tooth decay and to the best of our knowledge, this is the first public report that has quantify the biofilm forming potential of *C. albicans* in Central Nigeria. This may improve the current knowledge of microbial pattern and biofilm forming potentials in tooth decay individuals in Nigeria.

KEYWORDS: Biofilm; Tooth decay; Dental plaque; Microorganisms; ELISA; Central Nigeria.

1.0 INTRODUCTION

The oral cavity provides a diverse environment for the growth and colonization of a wide variety of microorganisms most especially the bacteria.[1] These microbes are ubiquitous entities in nature as such can be found on human and animal bodies as normal flora. These microorganisms can be planktonic or sessile and frequently organize themselves into a consortium referred to as "biofilm" that adheres to favorable animate or inanimate surfaces, interacts and produces extracellular matrix.[2,3]

Dental plaque is a general term for the diverse microbial community (predominantly bacteria) found on tooth surfaces, embedded in an exopolymeric substances of bacteria and salivary origin.[4] It is a sticky colorless deposit at first, but when it forms tartar, it is often brown or pale yellow. It is commonly found between the teeth, on the front of teeth, behind teeth, on chewing surfaces, along the gum-line, or below the gum-line cervical margins.[5] Bacterial plaque is one of the major causes for dental decay and gum disease.[5,4] Dental caries is a standout amongst the commonest disease around the world. Approximately 2.4 billion individuals (36% of the global population) have permanent teeth rotting. The disease affects 620 million children or 9% of the world's population. It also influences 60 – 90% of school youngsters and most adults.[6]

The major features that distinguish biofilm forming bacteria from their planktonic counterparts are their surface attachment ability, high population density, extracellular polymeric substances (EPS) slime and a wide range of physical, metabolic and chemical heterogeneities.[7]

Progression and build-up of oral biofilm can give rise to a localized destruction of the tissues of the tooth by acids produced from the bacterial breakdown of fermentable sugar known as tooth decay and periodontal problems such as gingivitis and periodontitis.[8] Dental hygiene therefore is important as dental biofilms may become acidic causing demineralization of the teeth or harden into dental calculus known as tartar.[9]

Persistent dental disease is painful, and most importantly, it has also been suggestively linked to diabetes, high blood pressure, heart disease, and multiple sclerosis in life. The pain can be worsened by heat, cold, or sweet food and drinks.[10,11] Dental caries can also cause bad breath and foul tastes. In highly progressed cases, infection can spread from the tooth to surrounding soft tissues which may lead to an edentulous mouth.[11]

Oral biofilms mostly consist of multiple bacterial strains which include mainly the *Streptococcus* sp. and *Lactobacillus* sp.[3,12] *Candida albicans* is the major fungal agent associated with the oral cavity of 20-40% of healthy individuals.[1] In the presence of sugar, these bacteria ferment the dietary sugars producing acid and lowering the pH of the mouth, leading to demineralization of the teeth which is the initial step in oral biofilm formation.[3] With the dearth of data on the assessment of biofilm forming potentials of microorganisms associated with tooth decay in Nigeria, this study was setup to isolate and identify common microorganisms associated with tooth decay and assessing their biofilm forming potentials in a tertiary healthcare facility in Central Nigeria.

2.0 MATERIALS AND METHODS

Sample Collection

The swab samples were collected from eleven (11) participants attending Federal Medical Centre, Keffi who agreed to participate in the study irrespective of their ages. The swabs were taken from the entire teeth surfaces, gingival, subgingival and buccal cavity. The samples were transported aseptically to the Microbiology Laboratory of Nasarawa State University, Keffi. The swabs were inoculated in Nutrient broths (TitanBiotech Ltd) and were incubated for 24 hours at 37°C.

Isolation of Microorganisms

The overnight broth cultures were standardized and streaked on nutrient agar plates and were incubated at 37°C for 24 hours. The random colonies were selected independent of their colony characteristics and streaked on differential and selective media such as Eosin Methylene Blue (HiMedia Lab), MacConkey agar (Sigma-Aldrich), Manitol Salt agar (HiMedia Lab), Sabouroud Dextrose agar (HiMedia Lab) and Blood agar (Titan Biotech Ltd). Pure cultures were obtained by frequent sub-culturing which were later maintained on the Nutrient agar slants at 4°C. Isolates were characterized culturally and morphologically by their appearance on agar plates and by Gram's staining.

Biochemical Characterization of the Isolates

The isolates were preceded for biochemical characterization including Indole test, Methyl red test, Voges-Proskauer test, Citrate test (IMViC test), catalase test and sugar fermentation tests and the results were compared with that of the already known species.[3]

Determination of Biofilm Formation by the Isolates Microtiter Plate Assay

The quantitative estimation of the biofilm formation was performed by the microtiter plate and spectrophotometer assay as described by.[13,3] The isolates were grown in Mueller-Hinton broth (MHB) and incubated overnight at 37°C in a static condition to obtain sufficient microbial growth. After 24 hours incubation, the cultures were standardized with the same medium and 150µl of each of the cultures were inoculated in the 96 well plates (Linbro®) in triplicates. The 96 well plates were then incubated at 37°C for 48 hours in a static condition. Contents of the wells were gently poured off in the laboratory sink after the incubation period and the wells gently washed in three trays of phosphate buffer saline (PBS pH 7.3) to remove free-floating planktonic cells. The plates were then stained with 0.1% (w/v) crystal violet (CV) solution and allowed to stand for 15 minutes. The CV was poured off and the plates were allowed to dry in an inverted form. The wells of the microtiter plates were destained with 150µl of 95% ethanol-acetic acid and allowed to stand for 15 minutes. Enzyme Linked Immunosorbent Assay (ELISA) auto plate reader (Labsystems Type 355) of the Brucellosis Division of National Veterinary Research Institute (NVRI) Vom-Jos, Nigeria was used to measure the Optical density (OD) at wavelength of 620 nm. These OD values were considered as an index of attachment to surface. The experiment was performed in triplicates and the mean values were considered as an index of biofilm formation.

3.0 RESULTS

Out of the 11 swab samples analyzed, the microorganisms isolated based on age of participants is shown in Table 1. Table 2 shows the cultural, morphological and biochemical characteristics of the bacterial species isolated while Table 3 depicts the fungal specie isolated morphologically and using sugar utilization test.

The percentage of occurrence of the isolates is shown in Figure 1 while the result of the biofilm formation from the selected isolates is shown in Table 4.

Table 1: Isolation of Microorganisms Associated with Tooth decay.

Age (Years)	Samples collected	Media used	Microorganism Suspected
≤10	TD1	MCA	*Pseudomonas* sp *Escherichia* sp *Proteus* sp
11-20	TD2	MSA, BA, EMB	*Staphylococcus* sp *Streptococcus* sp *Escherichia* sp
21-30	TD3/TD4/TD5	MCA, BA, SDA, MSA	*Streptococcus* sp, *Proteus* sp, *Lactobacillus* sp, *Pseudomonas*, Bacillus sp, *Lactobacillus* sp, *Streptococcus* sp, *Candida* sp, *Staphylococcus* sp
≥30	TD6	BA	*Lactobacillus* sp

Key: TD = Tooth decay, MCA = MacConkey agar, MSA = Mannitol salt agar, BA = Blood agar, EMB = Eosine methylene blue, SDA = Sabouroud dextrose agar

Table 2: Cultural, Morphological and Biochemical Characteristics of the Bacterial Isolates associated with tooth decay.

Cultural	Morphological		Biochemical tests							Inference
	Shape	Gram staining	IND	MR	VP	CT	OXD	CAT	GLU	
Pinkish colouration On MCA and greenish Metallic sheen on EMB	Rods	-	+	+	-	-	-	+	+	*Escherichia coli*
Circular, semi-transparent colonies with an area of clear haemolysis (0.5-1mm)	Cocci	+	-	-	+	-	-	-	+	*Streptococcus mutans*
Colourless colonies on MCA and purple on EMB	Rods	-	-	-	-	+	+	+	+	*Pseudomonas aeruginosa*
Pink colonies on MCA, Colonies with yellow zones on MSA	Cocci	+	-	+	+	+	-	+	+	*Staphylococcus aureus*
Beta-haemolysis on BA and appears flat, grey and dry	Rods	+	-	-	+	+	+	+	+	*Bacillus subtilis*
Swampy growth in the form of uniform film on MCA	Rods	-	-	+	-	+	-	+	+	*Proteus mirabilis*
Appears slightly opalescent and orange in colour on MRS agar	Rods	+	-	-	-	-	-	-	+	*Lactobacillus acidophilus*
Key: IND = Indole test, MR = Methyl red test, VP = Vogues Proskauer test, CT = Citrate test, OXD = Oxidase test, CAT = Catalase test, GLU = Glucose test, + = Positive, - = Negative, MRS = de Man, Rogosa and Sharpe Agar										

Table 3: Morphological and Sugar Utilization Test for Identification of Fungal isolate associated with tooth decay.

Morphology		Carbohydrate Assimilation Test							Presumptive
Gram staining	Shape/Size	TRE	XYL	GLU	GAL	MAL	LAC	SUC	identification
+ yeast	Oval, 4-6µm	+	+	+	+	+	-	+	*C. albicans*

Key: TRE = Trechalose; XYL = Xylose; GLU = Glucose; GAL = Galactose; MAL = Maltose; LAC = Lactose; SUC = Sucrose; + = Positive, - = Negative; µm = Micrometer, *C. albicans* = *Candida albicans*

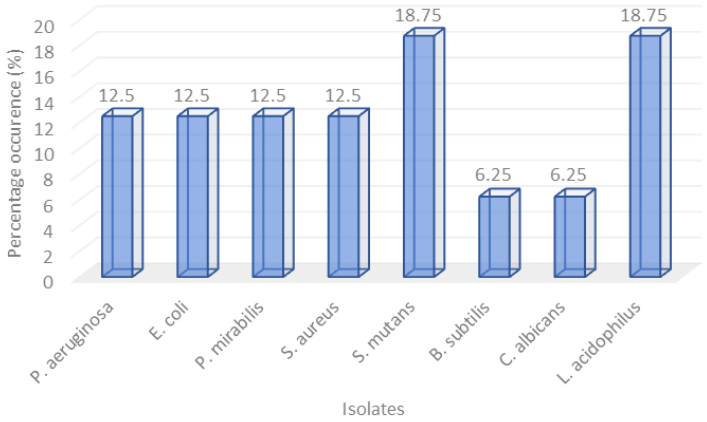

Figure 1: Percentage Occurrence of isolated microorganisms associated with tooth decay

Table 4: Results of Biofilm Formation of the selected isolated microorganisms associated with tooth decay

Isolates	Samples isolated	OD at 620nm			Mean OD value	Biofilm strength
		OD1	OD2	OD3		
P. aeruginosa	TD1	0.445	0.418	0.418	0.432	S
P. aeruginosa	TD5	0.245	0.203	0.209	0.215	M
E. coli	TD1	0.507	0.507	0.533	0.512	S
E. coli	TD2	0.134	0.126	0.143	0.130	W
P. mirabilis	TD1	0.215	0.207	0.225	0.212	M
P. mirabilis	TD4	0.219	0.120	0.301	0.316	M
S. aureus	TD2	0.137	0.141	0.139	0.135	W
S. aureus	TD5	0.121	0.142	0.105	0.119	W
S. mutans	TD2	0.215	0.202	0.223	0.209	M
S. mutans	TD3	0.372	0.302	0.311	0.324	M
S. mutans	TD4	0.486	0.419	0.422	0.438	S
B. subtilis	TD5	0.259	0.257	0.257	0.254	M
C. albicans	TD4	0.496	0.352	0.517	0.451	S
L. acidophilus	TD3	0.224	0.321	0.194	0.242	M
L. acidophilus	TD5	0.324	0.321	0.194	0.276	M
L. acidophilus	TD6	0.210	0.265	0.233	0.232	M
Control	-	0.001	0.010	0.001	0.004	-

Key: OD = Optical density; nm = nanometer; S = Strong; M = Moderate; W = Weak; N = Non-biofilm formers

4.0 DISCUSSION

Dental decay is one of the most common and less attended diseases in the developing countries. There are myriad of human pathogenic agents that dwell in the oral cavity of man with peculiarity of oral flora among individuals. Microbial strains isolated and identified from tooth decay participants based on their ages showed *S. mutans* and *L. acidophilus* (18.75%), *P. aeruginosa, E. coli, P. mirabilis* and *S. aereus* (12.5%) while *B. subtilis* and *C. albicans* (6.25%) percentage of occurrences. Several studies have reported similar microorganisms.[1,12,4,3] A related study reported that children aged 5-14 years old had *Streptococcus spp* as the commonest organism isolated in dental caries followed by *S. aureus.*[14] This present study agrees with a previous study who reported children aged 1-15 years with *S. mutans* as the most isolated pathogen followed by *Lactobacillus* and then *S. aureus. aureus* and *Candida spp* are part of the microbiota of the oral cavity and cause infections in that ecosystem.[15] Individuals most susceptible to Candida infections are diabetics, those with immunodeficiency (e.g., AIDS), catheterized patients, and those individuals who are taking

antimicrobial medications.[16] This might be why *C. albicans* was isolated from participants aged 21-30 years old in this present study.

The quantitative estimation of the biofilm was done by microtiter plate assay. Optical Density (OD) was recorded at 620nm using the ELISA auto plate reader (Labsystems Type 355) of the Brucellosis Division of National Veterinary Research Institute (NVRI) Vom-Jos Plateau state. The mean values of OD blank were subtracted from the mean values of OD of test strains. The isolates were classified into: non-adherent (OD < ODc); weakly-adherent (ODc < OD < 2xODc); moderately-adherent (2xODc < OD < 4xODc); strongly-adherent (4xODc < OD) (Table 4)

From the 16 isolates tested for biofilm forming potentials, 5(31.25%) were strong, 9(56.25%) were moderate and 2(12.5%) were weak biofilm formers respectively (Table 4). In this present study, *E. coli* isolated from age range 5-10 years showed the strongest biofilm potential (0.504), followed by *C. albicans* isolated from sample TD4 of adults with age range 21-30 years (0.435). *P. aeruginosa* and *S. aureus* isolated from children of age ranges 5-10 and 11-20 years had the same biofilm forming potential value of (0.432) respectively while the strain of *S. mutans* isolated from sample TD4 of age range 21-30 years also showed a strong biofilm forming potentials (0.403). All the strains of *L. acidophilus*, *P. mirabilis* and *B. subtilis* isolated from different samples across the age ranges were moderate biofilm formers while a strain of *E. coli* isolated from sample TD2 age range 11-20 years and *S. aureus* from TD5 age range 21-30 years showed weak biofilm forming potentials.

Dental biofilm can greatly affect the health of its host and lead to the development of many diseases. Understanding biofilm characteristics and its communities and ensuring proper control therefore will enable disease prevention. Dental plaque biofilm includes all of the characteristics of biofilm architecture and microbial community interaction, but, more specifically, it develops in the oral cavity, consist of more than 700 contributing oral microbial species and demonstrate a distinct method of conditioning the tooth surfaces.[17,18,19] Due to the highly specialized, coordinated, multi-species forms of microorganism found in dental biofilm life located permanently on the tooth surface in a matrix, surrounded by a layer of extracellular polysaccharides, this makes the microorganisms more resistant to immunological defense systems and less susceptible to antimicrobials.[17]

The oral cavity biofilm is a structured, three-dimensional ecosystem of microbial strains attached to the oral surfaces, which are found to be considerably on enamel. Those key microbes that contributes to the formation of biofilm in its early stage mainly include Streptococci, especially *S. mutans*, *S. mitis*, *S. gordonii*, and *S. anginosus* amongst others.[12]

A similar study reported that dental biofilm is the commonest dental disease in children, and this also leads to tooth loss in adults. Galleh *et al*. also reported in their study that *Pseudomonas aeruginosa* forms a strong biofilm which is resistant to most of the plant extracts used in their studies.[20,21,22] Risks factors such as diabetes, smoking, irregular tooth brushing etc were identified as some of the causes of dental caries. In diabetic patient, the high glucose levels in the saliva expand the measure of fermentable sugars by oral microorganisms, prompting to generation of acidic by-products that cause teeth demineralization in dental caries.[14] Every day smoking was connected with expanded utilization of sugar in tea or coffee, and with more continuous liquor utilization. It is likewise observed that smokers have insufficient brushing propensities than non-smokers.[23]

5.0 CONCLUSION

This study has reported the predominance of bacterial species (especially *S. mutans* and *L. acidophilus*) associated with tooth decay and to the best of our knowledge, this is the first public report that has quantify the biofilm forming potential of *C. albicans* in Central Nigeria. All the bacterial and fungal isolates identified in this study were reported to be involved in causing dental caries due to their biofilm formation ability on tooth surfaces. Probable factors such as diabetes, smoking, irregular tooth brushing, fermentable sugars in the oral cavity were identified as some of the causes of dental caries from previous studies. This may improve the current knowledge of microbial pattern and biofilm forming potentials in tooth decay individuals in Nigeria. Therefore, dental hygiene advocacy and sensitization is paramount to the reduction and possibly eliminating of microbial dental caries.

Limitation

Protein Expression and Purification, Cell Invasion Assay and Enzyme Activity Assay of the identified organisms were not performed due to limited resources and availability of modern research facilities. Further studies to investigate antibiofilm inhibitory effects of some plant extracts should be carried out since, plant materials were reported to have antimicrobial activities.

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