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Isolation and Identification of Bacteria Associated with Biofilm Production in University of Agriculture,

Makurdi, Benue State, Nigeria.

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Abstract
The study was carried out to isolate and identify some bacteria associated with biofilm production in University of Agriculture, Makurdi. Used toothbrushes, floor and sink swabs were collected and checked for biofilm formation. The isolates were identified using conventional methods. Three (3) bacteria were isolated and analysed namely; *Staphylococcus aeurus, Escherichia coli* and *Pseudomonas aeruginosa.* It was observed that the sink swab sample had the highest level of biofilm formation (43.48%) followed by the flour swab (30.43%) and lowest in tooth brushes (26.09%). However toothbrush sample from the female student had the highest level of biofilm.
Keywords: Biofilm, swab, tooth brush, *Staphylococcus aeurus, Escherichia coli* and *Pseudomonas aeruginosa.*

Introduction

Biofilm formation is a process whereby microorganisms irreversibly attach to and grow on a surface and produce extracellular polymers that facilitate attachment and matrix formation ,resulting in an alteration in the phenotype of the organisms with respect to growth rate and gene transcription [1][4]. Microbes form a biofilm in response to many factors [1][4][19], which may include cellular recognition of specific and non-specific attachment sites on a surface, nutritional cues, or in some cases by exposure of planktonic cells to sub-inhibitory concentrations of antibiotic,[8][13][17]. When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behavior in the large suite of genes are differentially regulated [27].

Biofilms is often similar to growth of microbes in colonies. Generally in colonies, the most rapid cell growth occurs at the colony edge [2] [25]. Growth is much slower in the centre, and cell autolysis takes place in the older central portions of some colonies[29][30]. These differences in growth are due to gradient of oxygen, nutrients and toxic products within the colony [30]. The size and shape of a colony depends on many factors. Nutrient diffusion and availability, bacterial chemo taxis, and the presence of liquid surface, all appear to play a role in pattern formation. Cell-cell communication is important as well [24]. The final stage of biofilm formation is known as development and is the stage in which the biofilm is established and may only change in shape and size [22]. The development of a biofilm allows the cells inside to become more resistant to antibiotics administered in a standard fashion [18].

Biofilm pose a serious problem for public health because of the increased resistance of biofilm associated microorganisms to antimicrobial agents and the potentials for this microorganism to cause infections in patients with indwelling medical devices [1][4]. An appreciation on the role of biofilm in infection should enhance the clinical decision-making process. New strategies based on better understanding and isolation of some common biofilms in University of Agriculture Makurdi is necessary for public health purposes. Luckily, some biofilms also offer opportunities for positive industrial and environmental effects such as bio-remediating hazardous waste sites, bio-filtering industrial water and forming bio-barriers to protect soil and underground water from contamination [1][4].

Materials and Methods

Collection of Sample

To obtain the toothbrush scrapings, 10 new brushes were randomly distributed to 10 selected students comprising of male and female of different of groups in the ratio 1:1, they were instructed to use the brushes for one month, after which the brushes were collected and analyzed for biofilm formation.

Floor and Wash Hand Junks

Floor swabs of the Federal University of Agriculture Makurdi were collected from the clinic, also swabs of wash hand sinks were obtained using swab sticks and were collected at 2 weeks interval to allow for proper processing of samples after which they were all screened for biofilm formation [17].

Processing of Sample

After the collection of samples, the toothbrushes, swab samples were processed. The top of each brush were aseptically removed with a sterile razor and the remaining part of the brush head were immersed into a big test tube containing phosphate buffer solution for 10 minutes so as to allow removal of any sediment that has

adhere to the brushes for each sample respectively. Thereafter, the solution was properly mixed and a loopful of it was used for culturing [17].

Isolation of Bacteria Associating Biofilm from Tooth brushes

The streak plate method was employed. One loopful aliquots of phosphate buffer solution containing the sample from the toothbrushes was cultured on Blood and Mac Conkey agar plates. The plates were incubated aerobically and anaerobically at 37ᵒᶜfor 24 hours [9].

Floor and Wash Hand Junk

The junks and the floor swab were inoculated directly unto the 3 media namely; Blood Agar Macconkey Agar and Manitol-salt Agar and the cultures were incubated aerobically and anaerobically at 37ᵒᶜ for 24 hours.

Conventional Identification of Bacterial Associated with Biofilm formation

Overnight cultures on the plate were examined and identified based on colonial appearance, colonial morphology and the colour shown on different media. This was followed by Gram staining and the biochemical test on the isolates [9].

Grams Staining

A smear of each isolate was made on a clean grease free slide with the aid of a wire loop. This wire loop was used to fix and allowed to dry by passing the slide through the flame of a Bunsen burner.Crystal violet stain was flooded on the fixed smear and allowed to stay for 60 seconds. The stain was then washed off with clean water, then flooded with lugol's iodine for 60 seconds and decolorized rapidly with acetone-alcohol for 30 seconds. In a similar manner, the slide was counterstained with sefranin dye for 30 seconds. Which was washed off with clean water, the smear was then blot dried, the slide was examined under the microscope using 100X objective lens with immersion oil [9].

Biochemical Tests

Catalase Test

The catalase test was carried out in order to acertain the bacteria agent that possessed the enzyme catalase that breaks down Hydrogen peroxide to oxygen and water. The presence of catalase enzymes in the test isolate is detected using Hydrogen peroxide. 2-3ml of hydrogen peroxide solution was poured into a big test tube using a sterile wire loop or applicator stick , two colonies were picked and immersed in the hydrogen peroxide solution. Bubbles or froth formation was immediately observed in positive cases [9].

Oxidase Test

The oxidase test was carried out to identify organisms that produce the enzymes cytochrome oxidase. A piece of filter paper was used for the test. The filter paper was placed in a clean petri dish and 2-3 drops of freshly prepared oxidase reagent was added .Individual colonies were removed and transferred from the media plate using a sterile wire loop and smeared on the filter paper. An intensive purple colour was formed with 15 seconds over time due to the presence of oxygen in the air which indicates a positive test [9].

Coagulase Test

The test was used to identify *Staphylococcus aureus* which produces the enzyme coagulase. Two drops of distilled water was placed on a glass slide. The two water drops were emulsified with the test organism using a sterile wire loop. A loopful of plasma was then added and mixed well, then the slide was rocked gently for about 10 seconds .Macroscopic clumping indicates a positive test [9].

Antibiogram of Selected Bacteria Associated with Biofilm formation

Fifteen (15) different types of antibiotics disc were used, namely Streptomycin, Gentamycin, Ampicillin, Amoxil, Septrin, Chloramphenicol, Augmentin, Reflacin, Ceporex, Rifampicin, Norfloxacin, Levofloxacin, Tarivid, Nalidixic acid, and Ciprofloxacin. The diagnostic sensitivity testing agars were properly dried at 37ᵒᶜ to remove moisture. A colony of organism was streaked on the entire diagnostic sensitivity disc were placed at a distance away from each other. This was done with the use of sterile forcept. The plates were incubated at 37ᵒᶜ for 24 hours before reading the zone of inhibition. The zones of inhibition were measured in millimeter [17].

Statistical Analysis of Data

Chi-square was used to analyze the data obtained from the study using Statistical Package for Social Science (SPSS) VERSION 6.

Results and Discussion

Table1: Morphological and biochemical characterization of Bacterial Associated with Biofilm formation

Isolates Codes	Gram rxn	Catalase	Coagulase	Motility	Oxidase	Most probable bacterium	Colony appearance
A	+ve cocci in cluster	+	+	-	Not applicable	*Staphylococcus aureus*	Flat creamy shiny and yellowish colonies
B	-ve rod	Not applicable	Not applicable	+	-	*Esherichia coli*	Medium size, smooth round grayish-white colonies
C	-ve rod	Not applicable	Not applicable	+	+	*Pseudomonas aeruginosa*	Pale-pink colonies

Table 1 shows the characterization of biofilm forming isolate microscopic and biochemical characterization of bacterial isolates from specimen revealed the presence of *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeringinosa.*

Table2: Frequency of Biofilm formation in relation to Bacteria Isolates

Sample	No. of sample collected	No. of sample with growth of organism	No. of biofilm producing organism	% Biofilm
Tooth brush scraping	10	8	6	26.09%
Sink swab	10	10	10	43.48%
Floor swab	10	9	7	30.43%
Total	30	27	23	100%

The frequency of biofilm formation in specimen collected is shown in Table 2. A total of 27 bacterial isolate belonging to three genera, 23(100%) produce biofilm. The highest number of biofilm producing isolates was from sink (39.13%) and floor swabs (34.78%).

Table3: Distribution of bacterial isolates in tooth brush Biofilm formation with respect to Age Isolates in Biofilm

Age	No. of biofilm	*S.aureus*	*E.coli*
18-22	3(50%)	2(40%)	1(100%)
23-27	2(33.33%)	2(40%)	0(0)
28-32	1 (16.67%)	1(10%)	0(0.0%)
33 Above	0(0.0)	0(0.0)	0(0.0)
Total	6	5	1

Distribution of bacterial isolates in tooth biofilm with respect to age is shown in Table 3. Out of 6 isolates from brush that formed biofilm, the highest 3(50.0%) consisting of 2 staphylococcus aureus and E. coli were within age group 18 – 22 while the least (16.67%) was recorded within the age bracket 28 – 32.

Table4: Antibiogram of *S.aureus* Associated with Biofilm formation

Antibiotic	Sensitivity	Prevalence (%)
Ciprofloxacin	11	14.86%
Gentamycin	9	12.16%
Ampicillin	4	5.41%
Streptomycin	6	8.11%
Chloramphenicol	14	18.92%
Tetracycline	4	5.41%
Amoxil	6	8.11%
Rifampicin	5	6.77%
Norfloxacin	11	14.86%
Levofloxacin	4	5.41%
Total	**74**	**100%**

The antibiogram of biofilm forming *Staphylococcus aureus* is as shown in Table 4. Out of ten types of antibiotic used 14(18.86%) strongly sensitive to chloramphenicol. While 11(14.86%) and 9(12.18%) were sensitive to norfloxacin , ciprofloxacin and gentamycin respectively. Ampicillin, streptomycin, tetracycline, amoxil, rifampicin and levofloxacin were moderately sensitive with 4(5.41%), 6(8.11%), 4(5.41%), 6(8.11%), 5(6.77%), 4(5.41%) respectively. Ampicillin tetracycline and levofloxacin recorded the lowest sensitivity rates.

Table5: Antibiogram of Associated *Escherichia coli* with Biofilm formation

Antibiotic	Sensitivity	Prevalence (%)
Tarivid	12	15.58%
Reflacine	4	5.19%
Ciprofloxacin	10	12.99%
Augmentin	10	12.99%
Gentamycin	11	14.29%
Streptomycin	5	6.49%
Nalidixic acid	11	14.29%
Septrin	4	5.19%
Ampicillin	4	5.19%
Ceporex	6	7.79%
Total	**77**	**100%**

The Antibiogram of associated *Escherichia coli* with Biofilm formation is as shown in Table 5.Out of 10 types of antibiotic used 12(15.58%) E. coli showed strong sensitivity to tarivid, 10(12.99%) to ciprofloxacin and augmentin, 11(14.29%) to gentamycin and nalidixic acid. Also E.coli was found to be moderately sensitive to reflacine 4(5.19%), streptomycin 5(6.49%), septrin 4(5.19%), ampicillin 4(5.19%) and ceporex 6(7.79%).

Table6: Antibiogram of *Pseudomonas aeruginosa* Associated with Biofilm formation

Antibiotic	Sensitivity	Prevalence (%)
Tarivid	4	4.88%
Reflacine	10	12.20%
Ciprofloxacin	10	12.20%
Augmentin	5	6.10%
Gentamycin	4	4.88%
Streptomycin	10	12.20%
Nalidixic acid	14	17.07%
Septrin	10	12.20%
Ampicillin	5	6.10%
Ceporex	10	12.20%
Total	**82**	**100%**

The Antibiogram of *Pseudomonas aeruginosa* Associaed with Biofilm formation is as shown in Table 6. The organism was sensitive to reflacin 10(12.20%), ciprofloxacin 10(12.20%), streptomycin 10(12.20%), nalixidic acid 14(17.07%) and 10(12.20%) ceporex. It was observed to be moderately sensitive to tarivid 5(6.10%) augmentin 4(4.88%), ampicillin 4(4.88%) and tarivid 5(6.10%)

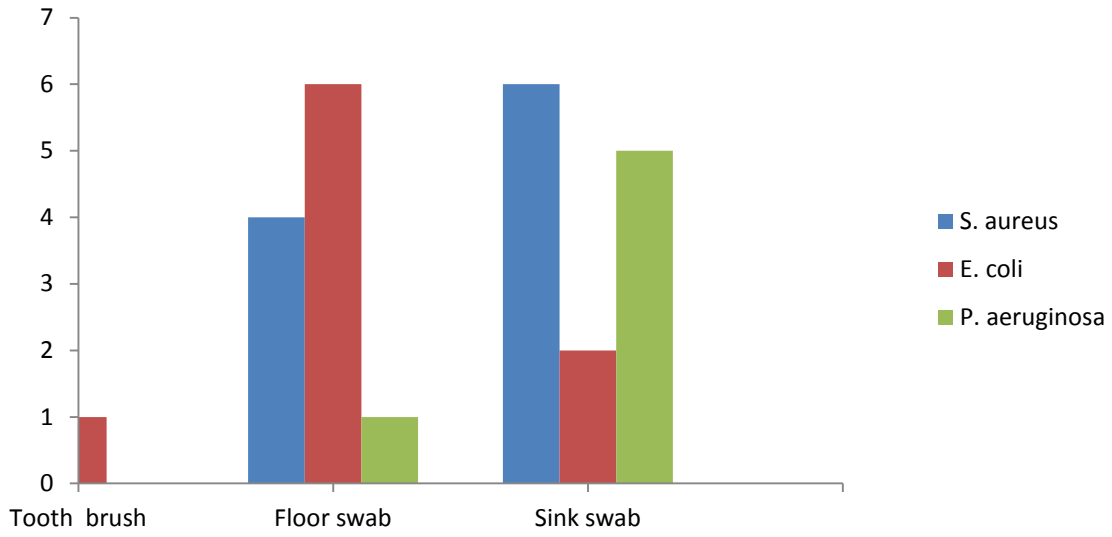

Fig 1: Distribution of Bacterial Isolates Associated with Biofilm formation

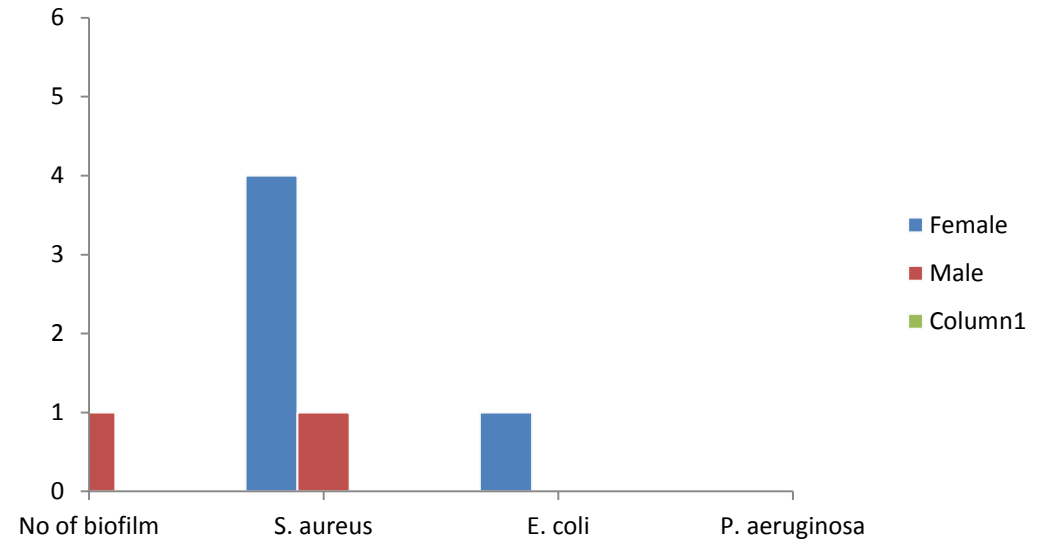

Fig 2: Frequency of Distribution of Bacterial Isolates in Tooth brush Associated with Biofilm formation according to gender

Discussion

The frequency of distribution of bacterial isolates associated with biofilm formation in this studied showed that isolate from sink were predispose to biofilm formation than tooth brushes and hospital floor. This is evidenced from the highest level (43.48%) of biofilm formation recorded in sink swab (junk) as against (30.43%) and (26.09%) for floor and tooth brushes respectively and not statistically significant at p>0.05. The observation in this study is consistent with other a finding [11] in which sink (junk) was reported to be a source of biofilm formation. The reason for higher colonization of biofilm forming bacteria in sink (junk) could be attributed to higher nutrients in the junk emanating from the remaining of waste products of water disposal through the sink. Microbial populations are found higher in almost all moist environments and this is an initial step in biofilm formation [13]. Accessing the distribution of bacteria isolates associated with the biofilm formation, the highest of the overall biofilm formation was due to the activities of *Staphylococcus aureus*, while the least was due to *Pseudomonas aeruginosa* [18] reported that surface polysaccharides play an important role in biofilm formation in *Staphylococcus aureus*. It is worthy of mention that many antibiotic employed in this study recorded partial or complete resistance against bacteria isolate associated with biofilm formation. The reason for this could be attributed to the formation of biofilm by the contribution of bacterial extracellular polysaccharide involved in biofilm formation as reported by [30]. The implication of the resistance could lead to prolong stay in the hospital exposing the patient to nosocomial infection [11]. The resistance could also lead to increase in socio-economic crisis when too much money is spent in buying drugs. The frequency of distribution of bacterial isolates associated with bioflm formation in tooth brushes according to gender and ages, gender (83.3%) was recorded among the females than those of male (16.67%) and was statistical significant (p>0.05). This could probably be attributed to the feeding habits of the students. Also occurrence of biofilm formation in females' toothbrushes was found to be higher than in their male counterparts, as the female students are used to sweet, candy, chocolate, chewing gum e.t.c this items are not common with the male [15]. The sugar deposit of this items can enhance the growth of micro-organisms thereby leading to plaque formation and hence infection. Similarly, this study revealed a higher prevalence among age bracket (18-22) years because they have more sugar in their diet at early age, inability to treat ailment due to low income and less education and was statistically not significant with respect to age (p>0.05).

Conclusion

From the study one can conclude that *Staphylococcus aureus* and *Escherichia coli* are associated with form biofilm on floor, toothbrushes and sink. It is very difficult to treat biofilm forming bacterial infection as a result of their ability to resist antibiotics. It is hoped that, the result of the research work will help in the control of biofilm formation especially among tooth brushes users in Federal University of Agriculture Makurdi Benue state.

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