DOI: 10.21088/jmrr.2395.6623.12126.1

Journal of Microbiology and Related Research
Volume 12 Number 1, January - June 2026

ORIGINAL ARTICLE

A Study on the Isolation and Identification of Biofilm forming Oral Flora

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HOW TO CITE THIS ARTICLE:

Neha Chauhan, Pallavi Sharma, Shweta Gupta et. al, A Study on the Isolation and Identification of Biofilm forming Oral Flora. J Surg. Nurs. 2026; 12(1): 07-16.

ABSTRACT

Biofilm is a community of micro-organisms, such as bacteria, that adheres to surfaces and form a protective slimy matrix. Biofilms are the most complex structure of micro-organisms attached to the animate and inanimate objects such as- teeth, pipes, medical devices etc. These cells are frequently embedded within a self-produced extracellular polymeric substance (EPS). This extracellular polymeric substance protects the bacterial cells from environmental conditions. Physical properties of biofilms such as attachment, mechanical strength, and antibiotic resistance can be attributed to EPS matrix. Dental biofilm forming organisms are major sources of the oral diseases like disease of the gums that is initiated by subsets of microbes that colonize on or just below the gingival margin. Dental Biofilms are the most prevalent cause of oral diseases that colonizes in the gingival and sub-gingival regions of the mouth. The present study aims to isolate the biofilm forming bacteria from the oral cavity, their morphological characterization, biochemical identification and detection of biofilm by various assays. Firstly, samples were collected from the oral cavity of healthy individuals, and then inoculated on various media such as- NA, TSA, and MS-Agar for isolation of biofilm forming microorganisms. Ten isolates were Gram positive cocci either in chains or clumps. Seven isolates were Gram negative rods and three isolates were Gram positive cocci in bunches. The isolates were characterized by Gram staining, Biochemical tests involving IMViC test, catalase test, oxidase, urease, sugar fermentation and motility test. The isolated organisms were observed for their ability to lyse the RBCs by haemolysis test. Biofilm formation assay was carried out by tube assay, tissue culture plate assay, coverslip assay and by microtiter dish assay. It was observed that out of 20 samples 14 samples were found to produce biofilm. Four out of fourteen isolates were strong and six isolates were moderate biofilm producers (10) by tube assay.

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➢ **Received:** 13-04-2026 ➢ **Accepted:** 17-05-2026

Five out of fourteen isolates were strong and six were moderate biofilm producers (11) by tissue culture plate assay and microtiter dish assay. While only very few samples showed the same formation by coverslip assay. Therefore, the TC plate assay and Microtiter dish assay can be used upon the tube method for the detection of biofilm formation.

KEYWORDS

• Biofilm Formation • Dental Plaque • Exopolysaccharides • Dental Flora • Tube Method • Adhesion Assay • Coverslip Assay

INTRODUCTION

Biofilms are masses of micro-organisms that bind to and multiply on a solid surface, typically with a fluid bathing the microbes. The biofilm forming bacteria are planktonic in nature i.e.; those that are not attached but are free-floating in an aqueous environment. Biofilm can be found almost everywhere associated with moisture.[1] Biofilms are agglomerates of microorganisms (bacteria, algae & fungi) that adhere to biological and non-biologicalsurfaces and are functionally organized in layers.[2] Biofilms are ubiqui-tous; they form on every surface wherever you find a combination of moisture, nutrients, you are likely to find biofilm.[3] Microorganisms require a humid environment to resist dehydration, for biofilm formation.[4] These planktonic or free living micro-organisms forms a community of microbes called biofilm that adheres to surfaces and form a slimy protective layer. These cells attached on surfaces via a self-produced extracellular polymeric substance (EPS). In addition to mechanical stability, biofilm formation stimulates a synergistic interaction that ensures survival in periods of starvation and prevents the displacement of extracellular enzymes.[5] They adhere to oral surfaces and are responsible for cariogenic action that leads to tooth decay and other periodontal diseases. The development of these highly ordered multicellular communities is a complex multistep process.[6] During biofilm formation, the bacterial cells transform from planktonic life to an immotile life form[7], resulting in a complex community consisting of different microbial subpopulations. Biofilm formation is initiated with the adhesion of planktonic bacteria to biotic or abiotic surfaces, the development of microcolonies, and a successive production of an extracellular matrix composed of polymeric substances such as proteins, polysaccharides, and extracellular DNA.[8] Three-dimensional structures develop through maturation and finally result in the detachment of single bacterial cells.[9] Microbial cells in biofilm experience impaired diffusion of nutrients and waste products with less nutrient availability in the core of the biofilm.[10] In addition, due to increased endogenous oxidative stress within biofilms, microbial cells are subjected to spontaneous mutations.[11] Genetic variations then give rise to microbial subpopulations that are physiologically heterogeneous.[12,13] Biofilm play a prominent role in human infections. More than 80% of microbial diseases have been linked to biofilm formation. Bacterial biofilms are involved in infection of the urinary tract, the female genital tract, the bloodstream, and the upper respiratory tract.[14-16] Oral biofilms can further affect the tissues surrounding the tooth where they cause inflammatory processes of the gingival, and when becomes persistent, cause damage to the alveolar process result in tooth loss.[17] 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.[18] The oral cavity is one of the most numerous in terms of bacterial species diversity microbiome of the human organism. Microbiome of the human oral cavity consists of difficult-to-determine number of microbiota representing anatomically limited distinct ecological niches, e.g., microbiota of the surface of the tongue, cheek, teeth, palate, gums, gingival pocket, etc. Except for the anatomical structure, the factors determining the variable composition of particular microbiota of the oral cavity are as follows: variable quality of saliva, being a natural protective barrier ensuring the maintenance of proper condition of the oral cavity, and also habits of diet and hygiene.[19] Mutans streptococci (*Streptococcus mutans*

and *Streptococcus sobrinus*) are the most chief bacteria in the pathogenesis of dental caries because of many epidemiological, experimental and animal studies. This is due to their ability of quick lactic acid formation from dietary carbohydrates, mainly sucrose and glucose.[20,21] The pathogenecity of these species is associated with their ability to form biofilms on solid surfaces. This feature allows microorganisms to form 3D structures in which cells become more resistant to antibiotics and changing environmental conditions.[22] *Streptococcus* mutans has major role in formation of dental caries.[18] After unavoidable pellicle formation, streptococci bind to the exposed proteins on the enamel surface. At physiological pH, pellicle proteins in the oral cavity are negatively charged.[23] Generally, the negatively charged bacterial cell membrane is more prone to adhere to positively charged surfaces than to negatively charged or uncharged surfaces.[24,25] *S. mutans* proteins involved in biofilm formation include glucan-binding proteins, collagen-binding proteins, glucosyltransferases, and the cell surface protein antigen c (PAc).[26]

The aim of this study is the isolation and characterization of dental biofilm forming microbes from healthy individuals. The isolates were further characterized by various biochemical tests and their ability to form biofilm was observed by tube assay, TC plate assay, coverslip assay and microtiter dish assay.

MATERIALS AND METHODS

Sample collection

A total of 20 oral cavity samples were collected from the students of University of Kota Campus, Kota Rajasthan, which includes both male and female population. Samples were collected using sterile swabs dipped in saline water. The swabs were taken from the complete teeth surface including gingival, sub-gingival and buccal cavity and transferred to laboratory for further analysis.

Isolation and Identification of biofilm forming microorganisms

A range of media were used for the proper isolation of oral flora. Such as Nutrient Agar (NA), Trypticase Soy Agar (TSA), and Mitis Salivarius Agar (MS-Agar). The swabs were directly inoculated on Nutrient Agar plates (HIMEDIA) and incubated for 24 hours at 37°C. The presences of creamy, pin-point, greenish colonies were observed on the Nutrient Agar plates after 24 hours. The random colonies were selected independent of their colony characteristics and pure cultures were obtained by frequent sub-culturing on the surface of TSA & MS-Agar plates for further purification and incubated at 37°C for 48 hours.

Haemolysis test

Blood haemolysis test was conducted to evaluate the extent of haemolysis, which is the destruction of red blood cells (RBCs). For this process Blood agar (Blood agar base supplemented with haemolytic powder) was prepared and the sample was inoculated on it using sterile inoculating loop. After inoculation the plates were incubated for 24 hours at 37^0 C. The very next day the plates were observed for α, β and γ haemolysis.

Biochemical Characterization

The isolates were progressed for Biochemical characterization including Catalase, Oxidase, IMViC test, urease test, motility test and Carbohydrate fermentation tests.

Detection of biofilm formation

Detection of biofilm formation was observed by tube assay and tissue culture plate assay, coverslip method and microtiter dish assay.

Tube assay

Tube method was performed as described by Christensen *et al.*[27] for qualitative estimation of biofilm production. The isolates were inoculated in 5ml of Trypticase soy broth and incubated for 72 hours at 37°C. After incubation the tubes were poured off and washed with Sodium Dodecyl Sulfate (SDS) and left to dry at room temperature. After that the tubes were stained with 0.1% Crystal violet. Excess stain was removed by washing the tubes with water or decolorizer. The tubes were placed upside down and then observed for biofilm formation. Formation of biofilm was confirmed with the presence of visible film on the wall and bottom of the tube.[18,28] The liquid interface did not indicate biofilm formation.

Tissue culture plate method

Tissue culture plate method was performed as described by Christensen *et al.*, 1985[27] for quantitative measurement of biofilm

formation. For this method a tissue culture plate made of polystyrene is used. A single colony from each sub-cultured plate on MS-Agar was inoculated in Trypticase soy broth. The tubes were incubated for 24 hours at 37°C. After the incubation, about 7 ml from each of the inoculated TSB tubes were aseptically transferred in the wells of a flat-bottomed six well TC plate, and the plate was incubated overnight at 37°C. Next day, the contents were discarded by inverting the plate. The TC plate was washed once by adding SDS into each well for 10 minutes and then the SDS solution was discarded. This was followed by adding 2 ml crystal violet (0.1%) to each well for biofilm staining. After 10 minutes the stain was removed and the plate was washed with water. Finally, the plates were left to dry at room temperature.

Coverslip method

Biofilm formation was also examined using the coverslip assay. The biofilm adhered to the coverslips were visualized under light microscope[29] In this assay, the cultures were inoculated into TSB and incubated for 24h at 37^0 C. After the incubation, about 7 ml from each of the inoculated TSB tubes were aseptically transferred into the wells of a flat-bottomed six well TC plate containing coverslips at the centre of each well. The plates and the coverslips were UV sterilized for 15-20 minutes. Plates were covered and placed in an incubator for overnight incubation at 37^0 C. The very next day the coverslips were dipped in 0.1% crystal violet for 15 minutes in order to stain the bacteria which adhered on the coverslips. After 15 minutes the excess stain was washed with water and then it was left to air dry. The bacteria at the air-liquid contact on each coverslip were visualized under the microscope.

Microtiter dish assay

The microtiter plate method was used to screen the isolates for the capacity for producing biofilm. A single colony from each sub-cultured plate on MS-Agar was inoculated in a glass test tube containing 10 ml of TS broth. The tubes were incubated for 24 hours at 37°C in static condition. Each well of sterile, polystyrene, 96 well-flat bottom tissue culture plates was filled with 200µl aliquots of the diluted overnight cultures and broth without culture was used as control. The plate was incubated for 24 hours at 37°C to observe biofilm formation. Each well's content was carefully removed after the appropriate incubation time by gently tapping the plate. After that, the wells were washed with SDS to get rid of any planktonic bacteria that were floating around. After that, the plates were then stained with 0.1% (w/v) crystal violet solution. The plate was kept for 15-20 minutes to get the stain absorbed properly. After that, the excess stain was washed off thoroughly with SDS again and the plate was left to dry. The optical density (OD) was determined at 630 nm with a microtiter plate reader (Labsystems LDxR1). These OD values were deemed as a measure of biofilm formation and surface adhesions.[28,30]

RESULTS AND DISCUSSION

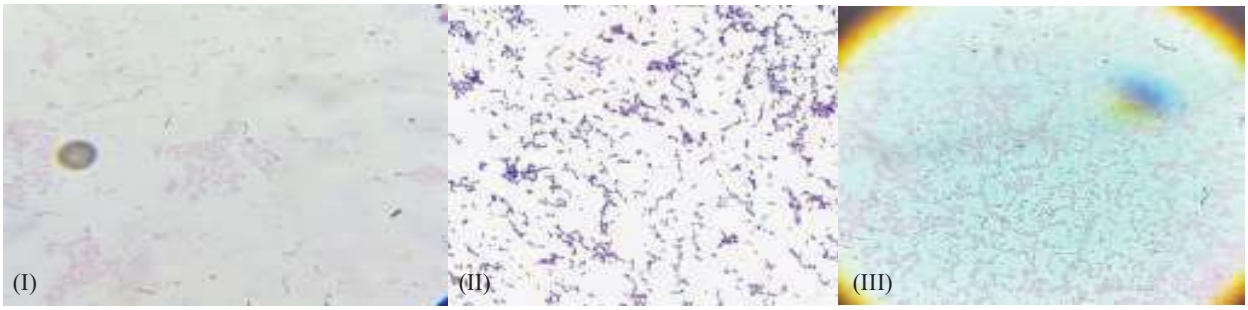
Morphological characterization

The 20 isolates were grown on NA was selected for characterization. Colony characteristics of six isolates (1, 2, 6, 9, 13, 14) out of 20 isolates shows small, round, smooth, and slightly raised and whitish colonies which may *Streptococcus spp.* Four isolates (3, 8, 10, 16) shows small to medium in size, circular, grayish-white, smooth, glossy colonies which may be *Streptococcus spp.* Four isolates (4, 5, 7, 19) shows Large, round, raised, smooth, mucoid, shiny and greenish colonies which may be *Pseudomonas*. Three isolates (11, 18, 20) shows smooth, circular, convex, and translucent colonies which is *E.coli.* While three isolates (12, 15, 17) shows medium, round, opaque, smooth, creamy, and golden-yellow colonies which may be *Staphylococcus spp.*

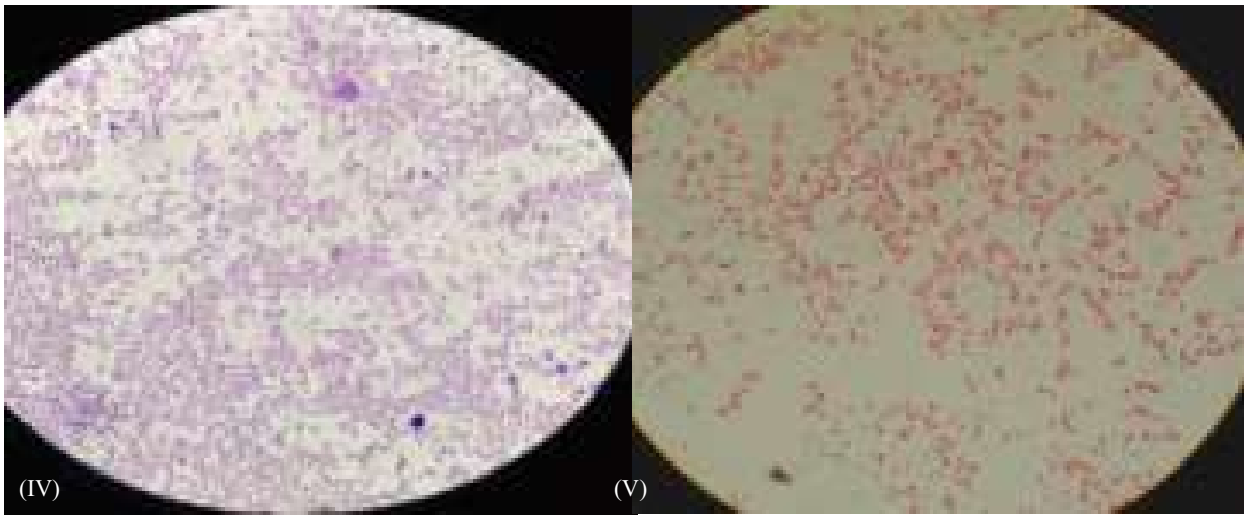
The microscopic observation revels that the ten isolates were Gram positive cocci either in chains or clumps. Seven isolates were Gram negative rods and three isolates were Gram positive cocci in bunches.

Blood Haemolysis

Blood haemolysis was observed on blood agar supplemented with haemolytic powder. Ten isolates which were *S. mutans* and *S. sanguis* shows α-haemolysis, seven isolates which were *P. aeruginosa* and *S. aureus* shows ß-haemolysis and three isolates which were *E. coli* shows no haemolysis or γ-haemolysis.



(I) Streptococcus mutans (II) Streptococcus sanguis (III) Pseudomonas aeruginosa



(IV) Staphylococcus aureus *(V) E. coli*

Figure 1: (I)-(V) Showing microscopical observation of the isolates

α-haemolysis β-haemolysis ϒ-haemolysis

Figure 2: Showing α, β, γ haemolysis

Biochemical characterization

The five bacterial species were biochemically identified to be - *Streptococcus mutans*, *Streptococcus sanguis, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Esherichia coli*. (Table 2). Three species *P.aeruginosa, E.coli, S.aureus* were positive for catalase test, while two species *S.mutans* and *S.sanguis* were negative for catalase test. Only one species Pseudomonas aeruginosa was positive for the oxidase test, while *Streptococcus mutans*, *Streptococcus sanguis*, *Staphylococcus aureus*, and *Esherichia coli* were negative for the oxidase test. The bacterial isolates were preceded for IMViC test; results are shown in table-2. Only *E.coli* gave positive indole test, due to presence of enzyme tryptophanase which convert tryptone from water into the indole, while *Streptococcus mutans*, *Streptococcus sanguis, Pseudomonas aeruginosa*, and *Staphylococcus*

aureus were negative for indole test. *E.coli* and *Staphylococcus aureus* were found positive for methyl red test due to the fermentation of sugars and production of acid in the medium and confirmed by the deep red color of the broth. *Pseudomonas aeruginosa*, *Streptococcus mutans*, and *Streptococcus sanguis* were negative for MR test. Three species *S. sanguis*, *P. aeruginosa*, *E. coli* were found negativefor VP test, while *S. mutans*, and *S. aureus* gave positive results due to the presence of acetoin in the medium. Only two species *P.aeruginosa* and *S. aureus* were found positive for citrate utilization test, the other three species *S. mutans, S. sanguis* and *E. coli* were citrate negative. Only one species that is S. aureus was found urease positive, while the other four species *S. mutans, S. sanguis*, P. aeruginosa, and *E. coli* were urease negative.

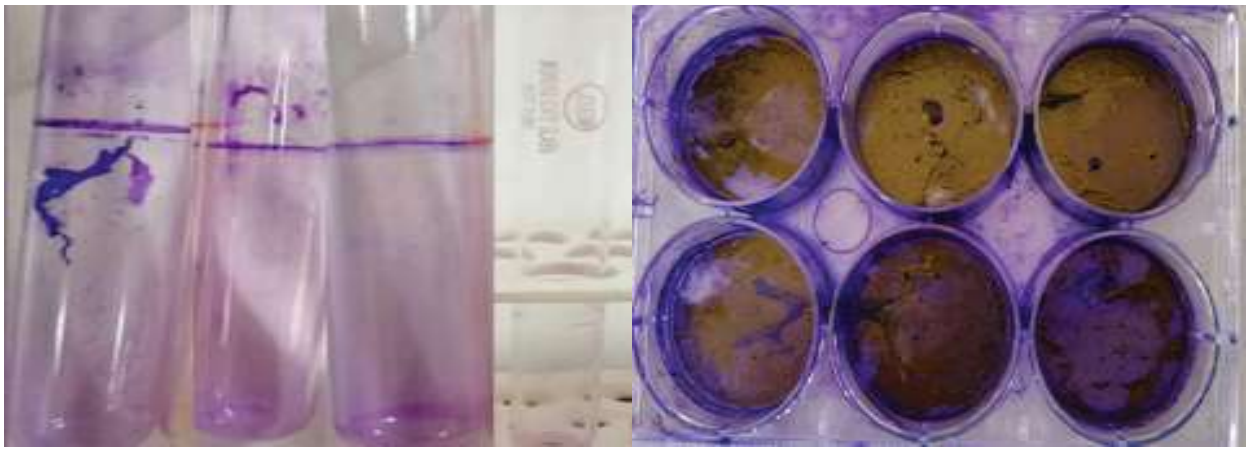


Figure 3,4: Showing biofilm formation by Tube assay and Adherent assay

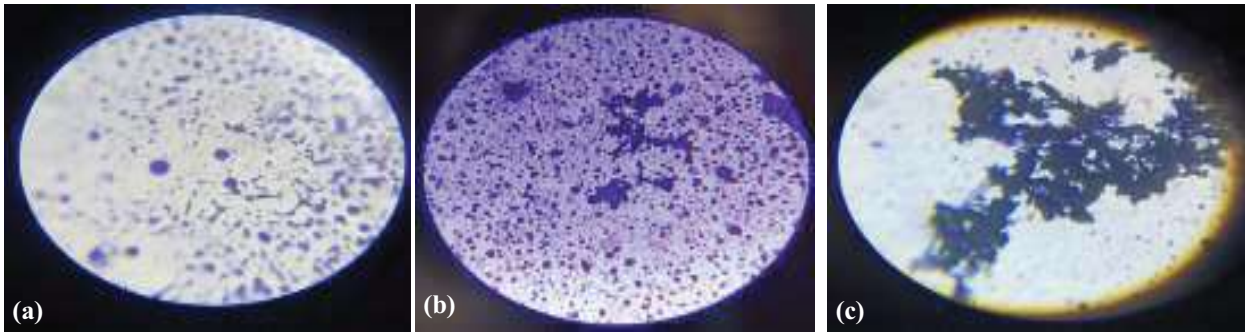


Figure 5: Showing (a) weak biofilm, (b) moderate biofilm, (c) strong biofilm by Coverslip assay

Table 1: Biochemical identification of the isolates (N=20)

Isolates	GS	Cat.	Oxi.	Indole	MR	VP	Cit.	Urease	Glu. A/G	Suc. A/G	Mann. A/G	Lac. A/G	Motility	Organisms
AG-1	+	-	-	-	-	+	-	-	+ +	+ +	+ +	+ +	NM	Streptococcus mutans
AG-2	+	-	-	-	-	+	-	-	+ +	+ +	+ +	+ +	NM	Streptococcus mutans
AG-3	+	-	-	-	-	-	-	-	+ +	+ +	+ +	+ +	NM	Streptococcus sanguis
AG-4	-	+	+	-	-	-	+	-	- -	- -	- -	+ +	M	Pseudomonas aeruginosa
AG-5	-	+	+	-	-	-	+	-	- -	- -	- -	+ +	M	Pseudomonas aeruginosa
AG-6	+	-	-	-	-	+	-	-	+ +	+ +	+ +	+ +	NM	Streptococcus mutans
AG-7	-	+	+	-	-	-	+	-	- -	- -	- -	+ +	M	Pseudomonas aeruginosa
AG-8	+	-	-	-	-	-	-	-	+ +	+ +	+ +	+ +	NM	Streptococcus sanguis
AG-9	+	-	-	-	-	+	-	-	+ +	+ +	+ +	+ +	NM	Streptococcus mutans
AG-10	+	-	-	-	-	-	-	-	+ +	+ +	+ +	+ +	NM	Streptococcus sanguis
AG-11	-	+	-	+	+	-	-	-	+ +	+ +	+ +	+ +	M	Esherichia coli
AG-12	+	+	-	-	+	+	+	+	+ +	+ +	+ +	+ +	NM	Staphylococcus aureus
AG-13	+	-	-	-	-	+	-	-	+ +	+ +	+ +	+ +	NM	Streptococcus mutans

table cont....

Isolates	GS	Cat.	Oxi.	Indole	MR	VP	Cit.	Urease	Glu. A/G	Suc. A/G	Mann. A/G	Lac. A/G	Motility	Organisms
AG-14	+	-	-	-	-	+	-	-	+ +	+ +	+ +	+ +	NM	Streptococcus mutans
AG-15	+	+	-	-	+	+	+	+	+ +	+ +	+ +	+ +	NM	Staphylococcus aureus
AG-16	+	-	-	-	-	-	-	-	+ +	+ +	+ +	+ +	NM	Streptococcus sanguis
AG-17	+	+	-	-	+	+	+	+	+ +	+ +	+ +	+ +	NM	Staphylococcus aureus
AG-18	-	+	-	+	+	-	-	-	+ +	+ +	+ +	+ +	M	Esherichia coli
AG-19	-	+	+	-	-	-	+	-	- -	- -	- -	+ +	M	Pseudomonas aeruginosa
AG-20	-	+	-	+	+	-	-	-	+ +	+ +	+ +	+ +	M	Esherichia coli

Table 2: Showing Biofilm formation by Tube assay, adherent assay, and microtiter assay

Isolates	Tube Assay	AdherentAssay	Microtiter Assay (OD at 630nm)
1	1	++	1.20
2	3	+++	3.16
3	0	-	0.63
4	0	-	0.82
5	2	++	1.72
6	1	+	1.09
7	1	+	0.63
8	2	++	2.65
9	3	+++	3.08
10	3	+++	3.10
11	0	-	0.75
12	2	++	2.39
13	0	-	0.73
14	2	++	3.25
15	3	+++	2.98
16	2	+++	2.57
17	1	-	0.49
18	0	-	0.65
19	2	++	1.98
20	0	-	0.6

Score-3, 3> OD> 2 (>3.32) = strongly adherent (+++); Score-2, 2> OD> 1 (1.16-3.32) = moderate adherent (++); Score-1, 1> OD > 0.5 (0.58-1.16) = weakly adherent (+); Score-0, ODC=OD < 0.5 (0.58) = negatively adherent (-).[31]

Carbohydrate fermentation test was performed for glucose, sucrose, lactose and mannitol production analyzing acid and gas production. This test was positive for *S. mutans*, *S. sanguis, S. aureus*, and *E. coli* as all sugars were fermented by these organisms with the change in color of broth from yellow to pink (acids production) was observed, except the 4 samples which were *Pseudomonas aeruginosa*, this bacteria was negative for three sugars, in which no fermentation of sugars and no color change was observed and was positive for only lactose. The isolates were tested to check whether they are motile or non-motile by motility test. This test was positive for E.coli with a peritrichous flagella and *P. aeruginosa* with a single polar flagellum and was negative for *S.mutans, S.sanguis*, and *S.aureus* with no flagella present.

Detection of biofilm formation

Tube Assay

The qualitative tube assay was visually performed to determine the ability of the isolates to form biofilm to the sides of borosilicate test tubes. Out of 20 samples, 14 isolates (70%) showed biofilm formation by tube assay, but out of them 4/14 (28.5%) were weak or poor biofilm producers, 6/14 (42.8%) were moderate biofilm producers and 4/14 (28.5%) were strong biofilm producers. The interpretation of this test was recorded as strong adherence 3 (+++),

moderate 2 (++), weak 1 (+) or negative 0 (-) (Figure 3 and 4 Table 2).

Tissue culture plate assay

The standard tissue culture plate method detected 13 out of 20 (65%) isolates were biofilm producers, but out of them 5/13 were strong biofilm producers, 6/13 were moderate biofilm producers and 2/13 were weak or poor biofilm producers. The interpretation of this test was recorded as strong (+++), moderate (++), weak (+) and negative (-) (Figure 4, Table 2).

Coverslip assay

At the air-liquid interface, a visible line of biofilm growing more than 2 mm was observed in the coverslip. Below this line, the coverslip was darkly discolored, indicating that 8/20 isolates (40%) had biofilm growth. the findings were interpreted as follows: no adherence, low level of adherence with only light staining and no-biofilm line at the air-liquid interface, intermediate level of adherence with staining below the air-liquid interface & high level of adherence with as seen under a microscope (Figure 5), a clearly defined line of staining at the air-liquid interface & staining across the lower half of the coverslip.

MICROTITER PLATE ASSAY

The quantitative microtiter plate adherence assay was predominantly evaluated by visual identification of the degree of adherence of the isolates to the bottom of each well. The Optical Density (OD) was recorded at 630nm with an ELISA reader. The cut off value was calculated by taking the average of negative control and by addition of 3 times of SD. The isolates were classified in the following manners: OD>4xODC were considered as strongly adherent (+++); 4xODC≥OD>2xODC was considered moderately adherent (++), 2xODC≥OD>ODC were considered weakly adherent (+) and those having ODC≥OD were considered as negatively adherent (-) whereas ODC was the cut off optical density[28] (Table 2).

The present study focuses on the isolation and characterization of the biofilm forming microorganisms from oral cavity of healthy individuals. The biochemical tests identified the following bacteria- Streptococcus mutans, Streptococcus sanguis, Pseudomonas aeruginosa, Staphylococcus aureus, and E.coli. The ability of these bacteria to form biofilm was checked by the qualitative and quantitative methods. E.coli was found to not form biofilm by these methods, while the other four bacteria forms weak, moderate, and strong biofilm. It was found by the present study that Streptococcus species is most dominating over other species.

CONCLUSION

It was concluded from the present study that microtiter dish assay and tissue culture plate assay is the most reliable method over the test tube assay and coverslip assay for the detection of Biofilm in oral isolates. Biofilm is the main cause of dental plaque and other oral diseases. Also the biofilm cause a multitude of problems in the field, like adhesions in the catheters or several diagnostic tools. A detailed study was needed up to the molecular level to find the genes involved in the formation of Biofilm.

Acknowledgement

The authors are highly grateful to the Department of Microbiology, UOK, Kota for the kind support.

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