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OPTIMIZATION OF CULTURE CONDITIONS FOR A BIOFILM FORMING BACTERIUM

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ABSTRACT

Oral hygiene is an essential component of human health. Dental caries is one of the most common diseases of man. Some specific species of bacteria are responsible for the formation of this disease. In the present investigation, biofilm forming organisms were isolated from dental caries affected man. The isolates were screened using test tube assay, glass cover slip assay and microtiter plate assay. Different conditions for better biofilm formation and antibiotic susceptibility of the selected organism were investigated. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was also determined. Among the selected isolates, *Streptococcus mutans* was found as better biofilm former that was finally selected for detailed study. Optimized culture conditions for biofilm formation were found 37ºC for 24 hrs of incubation with the medium pH 6.5 by the selected isolate. Glucose and yeast extract induced biofilm formation. It was found that the selected isolate was sensitive to ciprofloxacin and tetracycline; intermediate to chloramphenicol and gentamicin; and resistant to amoxicillin, ampicillin, penicillin, cefixime and erythromycin. Results of this study suggest reducing excessive consumption of sugary foods and drinks to prevent the occurrence of dental caries. In case of therapeutic use, antibiotics should be given depending on the basis of antibiotic susceptibility.

KEY WORDS: **Biofilm, Culture conditions, optimization, bacteria**

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INTRODUCTION

Dental caries is one of the most prevalent infectious diseases of humans. It is preceded by accumulation of dental plaque. Dental plaque is a complex biofilm formed on teeth surface composed of self-produced extracellular polymeric substances (EPS), mainly glucans[1]. The fermentation of dietary sugars by acidogenic oral microbiota plays key role in the development of caries. The dissolution of mineral in enamel and dentin due to acids released by these microorganisms cause carious lesions[2] and extra cellular polysaccharides such as glucan and fructan was shown repeatedly in clinical studies[3,4]. Antibiotic resistances by pathogenic microorganisms were recognized as an increasing global problem and have become an important factor to be considered in the treatment of infection. In this study, biofilm producing microorganisms were isolated from dental plaque. Among these a bacterial isolate *Streptococcus mutans* (K_{23}) was selected for further study investigating the different factors in biofilm formation and antibiotic sensitivity of it was assayed against different commercially available antibiotics.

MATERIALS AND METHODS

Sampling

Dental plaque was collected from eight outdoor patients at dental unit of Chittagong medical college hospital in Chittagong, Bangladesh, who were suffering from different stages of dental caries. Samples were collected with a sterile cotton swab saturated with sterile physiological saline. Then the cotton swab was poured into a screw-cap tube containing 10 mL sterilized physiological saline. The pour plate technique was applied for isolation of bacteria using nutrient agar (NA) medium.

Screening for biofilm formation

To assess the ability of biofilm formation by the isolate during its growth, the following three screening methods were performed.

Test tube assay

A qualitative and quantitative assessment of biofilm formation can be performed by this method. If an organism has the ability to adhere to a smooth surface with the development of a film of growth, it is considered as biofilm former. However, ring formation at the air-liquid interface is not considered indicative of biofilm formation[5]. Biofilm production in test tube was done by the modified method of Christensen *et al*.[5]. For the production of biofilm in test tube, 0.25 mL fresh inoculum was inoculated in a test tube containing 5 mL fresh Luria-Bertani (LB) broth and then incubated at 37ºC for 24 hrs in anaerobic condition. After incubation, biofilm formation assay in tubes was performed as O'Toole *et al.*[6], with the following modifications. The culture media were discarded from the test tube and rinsed with distilled water for two times then stained with 1% (w/v) crystal violet solution (5 mL) for 30 min. Excess stain was removed by rinsing with distilled water for two times and then 5 mL of 95% ethanol was added and allowed for 30 min. It solubilized the remaining crystal violet attached to the cells. Finally, the absorbance of dissolved crystal violet was measured by a spectrophotometer at 600 nm.

Cover slip assay

Biofilm production on cover slip surfaces was done by the modified method of Inoue *et al*.[7]. For the production of biofilm on the surface of the glass cover slip, 3 to 4 sterilized glass cover slips were taken in a sterile petri plate without over lapping and then 15 mL of sterile LB broth medium was poured into the plate then 1 mL freshly prepared inoculum was poured into the plate and incubated at 37°C for 24 to 48 h in anaerobic condition. Biofilm formation on glass cover slip surfaces was estimated by conventional crystal violet staining method (O'Toole and Kolter[8]. After incubation, the cover slips were taken from petri plates and rinsed with distilled water then stained with 95% ammonium oxalate crystal violet for 30 second. Again rinsed with distilled and then observed under

microscope (12.5×40). The images of biofilms were captured which were then analyzed by image processing technique. Image processing was done by the modified method of Shumi *et al*[9]. In this experiment, the software Image J (Image J 1.46r. Wayne Rasband, National Institute of Health, USA) was used for image analysis. The Image J software was set in the computer, the captured images were collected and the area of an images was selected and then color differences were analyzed; it gives the mean of dark spots in a pixel area. Several images were measured to justify the result.

Microtiter plate assay

The screening for biofilm formation in microtiter plate was done following the modified method of microtiter plate assay of Stepanovic *et al.*[10] . To assess the biofilm formation of the selected isolates, an overnight culture of each isolate was grown in LB broth medium, which was used as inoculum. Six wells of a row in a microtiter plate were selected for the isolate and 180µL LB broth was poured into each well. A row of wells was kept uninoculated to treat this as control. The plates were then incubated at anaerobic condition for 24 hrs at 37ºC. Then, the content of each well was aspirated, and each well was rinsed two times with 200 µL of sterile physiological saline. The remaining attached bacteria were fixed with 200 µL of 95% ethanol per well, and after 15 min plates were emptied and left to dry. Then 2% crystal violet was poured into the wells at the rate of 200µL/well for staining biofilms and allowed for 5 min. Excess stain was rinsed off by water. Biofilms become visible on the floor and the wall of the wells. Then the dye bound to the adherent cells was resolubilized with 200 µL of 33% (v/v) glacial acetic acid per well. The absorbance or optical density (OD) of each well was measured at 630 nm by using an ELISA reader. In order to compare the results, the adherence capabilities of testing isolates were classified into four categories as Stepanovic *et al.*[10]. Based on absorbance or optical density (OD) of tested isolate and optical density of control (OD_c) the categories are as follows: (1) $OD \leq OD_c$: Not a biofilm producer (2) $OD_c < OD \leq 2OD_c$: weak biofilm producer (3) $2OD_c < OD \leq 4OD_c$: moderate biofilm produce (4) $4OD_c < OD$: strong biofilm producer.

Identification of the isolate

On the basis of cultural, morphological and biochemical characteristics, the selected isolate was provisionally identified according to Bergey's Manual[11].

Optimization of different conditions for biofilm formation

In the present study, different factors and the effect different carbon and nitrogen sources in biofilm formation were studied.

Incubation time, temperature and pH

In these experiments, microtiter plates were prepared similarly as in the modified method of microtiter plate assay described in screening procedure. The results of incubation period (24, 48 and 72 h) temperature (4, 27, 37 and 45ºC) and pH (4.5, 5.5, 6.5, 7.5 and 8.5) were recorded.

Effect of different carbon source

To observe the effect of different carbon sources on biofilm formation of the isolate, different carbohydrates (starch, glucose, fructose, sucrose or lactose) were used with a minimal salt medium (KCl, 0.5 g; $NaNO_3$, 3.0g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2$, 0.1 g; KH_2PO_4, 1.0 g; $FeSO_4.7H_2O$, 0.01 g; distilled water, 1000 mL and pH, 7.2). The biofilm formation assay was performed as previously described modified method of microtiter plate assay. The final concentration gradient of each of the sugars became 180, 450, 900, 1350 and 1800µg/200µL.

Effect of different nitrogen source

To observe the response of biofilm formation of the isolate, different inorganic and organic compounds (beef extract, yeast extract, peptone, tryptone, KNO_3 or NH_4NO_3) were used as nitrogen source with minimal salt medium. The Biofilm formation assay were also performed as previously described modified method of microtiter plate assay. The final concentration

gradient of each of the nitrogen source became 180, 450, 900, 1350 and 1800µg/200µL.

In vitro evaluation of sensitivity assay of the selected isolate against some commercially available antimicrobial agents

Determination of antibiotic sensitivity pattern

The disc diffusion method[12] was used to determine the antibiotic sensitivity pattern of the isolate. In this experiment a battery of standard antibiotic discs {Ampicillin, 25 µg; Penicillin, 10 µg; Amoxicillin, 30 µg; Ciprofloxacin, 5 µg; Erythromycin, 15 µg; Cefixime,5 µg; Gentamicin, 10 µg; Tetracycline, 30µg ; and Chloramphenicol 30µg (Oxoid Co., UK)} were used. For a susceptibility test, 0.5 McFarland standards was used to adjust the inoculum density. In this experiment, *S. aureus* ATCC25923 was used as a standard organism.

Determination of MIC and MBC

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, and minimum bactericidal concentrations (MBCs) as the lowest concentration of an antimicrobial that will prevent the growth of an organism after subculture onto antibiotic-free media. Six commercially available antibiotics i.e. Phenoxymethyl-Penicillin, Amoxicillin Trihydrate, Tetracycline HCl, Ciprofloxacin, Erythromycin, Cefixime (Square Pharmaceuticals Ltd, Bangladesh) and a mouthwash solution (Chlorohexidine gluconate, Unimed & Unihealth Ltd, Bangladesh) were used for the determination of MIC and MBC The MIC was determined by broth micro-dilution method[13]. The broth micro-dilution testing was performed according to Clinical and Laboratory Standard Institute's (CLSI) directions. Fifty microliter of 2-fold LB broth medium was poured into each well of the 2nd column of a sterile 96-well microtiter plate and 50 µL of 1-fold LB broth medium (pH; 7.2) was poured into each other wells of the plate. Then 50 µL of stock solution of an antimicrobial agent was poured into the 2nd well of a row and mixed by steady circulation motions of micropipette tip. It resulted in 2-fold dilution. From this 50 µL suspension was poured into the 3rd well of the row and mixed which again produce another 2-fold dilution. In this way, the gradual 2-fold dilution was done up to the 11th well. From 11th well 50 µL, the suspension was discarded without further addition to 12th well.

Inoculation was done by pipetting 50 µL of inoculum suspension in each well of a microtiter plate following the direction of 12th well to 1st well of each row. This resulted in another 2-fold dilution. Thus, the concentration of antibiotics in the 2nd well reduced to 1024 µg/mL to 256 µg/mL. The 1st and 12th wells of each row were considered as positive control for growth of the isolate. Then the microtiter plates were incubated at 37ºC for 20-22 h. After incubation, the tested microtiter plates were assayed by tetrazolium salt[14]. To each well, 10 µL of sterile 2,3,5-triphenyltetrazolium chloride (0.5% w/v) solution was added. Cultures were then incubated at 37ºC for 24 h. A change in color from light yellow to red indicated growth of bacteria and the MIC was interpreted visually. The MIC was estimated by visual observation as the first dilution, which completely inhibits bacterial growth in LB broth medium. The main advantage of the 'Micro dilution' method for the MIC determination lies in the fact that it can readily be converted to determine the MBC as well. To perform this, 1 loopfull of suspension from each of the three wells containing the three lower concentrations (including MIC) of an antimicrobial agent that showed no visual growth was streaked on Mueller-Hinton agar and the plates were then incubated at 37ºC for 24 to 48 h. The plates were observed for bacterial growth. The highest dilution at which at least 99 % of bacteria were inhibited was considered as MBC.

RESULTS AND DISCUSSION

Isolation

A total of 23 isolates were isolated from dental eight outdoor patients and these isolates were labeled from KA_1 to KA_{23}.

Identification

In the present study, based on above screening result an isolate KA_{23} was selected for detailed study. The selected isolate (KA_{23}) was tested for their morphological, cultural and biochemical properties (Table 1). These characteristics were then compared with the standard description of "Bergey's Manual of Determinative Bacteriology, 8th and 9th ed [11,15] and found the isolate was belong to the genera *Streptococcus* and was provisionally identified as *Streptococcus mutans*.

Screening for biofilm formation

Test tube assay

In this experiment, the absorbance of the destained solution were measured spectrophotometrically and found the range from 0.73 to 1.89 at 600 nm (Table 2). The data of the test tube assay (both visual observation and absorbance) were compared among the isolates and found that eight isolates such as $KA_{1, 3, 4, 7, 13, 18, 22}$ and $_{23}$ were better biofilm former, which were selected for further study by cover slip assay and microtiter plate assay. These results suggested that there is a correlation between the absorbance of destained solution and of visual observation.

Cover slip assay

The eight selected isolates were subjected to biofilm screening by cover slip assay. After stained with ammonium oxalate crystal violet the formation of biofilm on glass cover slips were observed under the microscope. The photographs of the images were then analyzed by Image J software for the measurement of dark spots on a pixel area of the image. The dark spots represent the mass of biofilms. The measurement of mean per pixel area has a correlation with the abundance of biofilms on cover slip. The mean of dark spots per pixel area ranges from 2.24386E-05 to 6.23574E-05. The data of cover slip assay including both visual observation and mean per pixel area of dark spots was compared among the selected isolates and found that KA_{13}, KA_{22} and KA_{23} were found better biofilm former (Table 3).

Microtiter plate assay

In case of microtiter plate assay, the selected isolates were then screened for biofilm formation in polystyrene microtiter plate. The principle of microtiter plate assay has similarity to the test tube assay. However, it has some modification that fixation of biofilm with 96% ethanol in the wells of a microtiter plate and staining with 2% crystal violet and destained with 33% glacial acetic acid. An ELISA reader at 630 nm measured the absorbance or optical density (OD) of the destained solution. The absorbance found to range from 0.092 to 0.207 (Table 4). Based on absorbance, it was found that KA_{13}, KA_{22} and KA_{23} were moderate biofilm former.

Optimization of different conditions for biofilm formation

Incubation period

The selected isolate (*S. mutans*) showed optimum biofilm formation at 24 hrs of incubation. The of biofilm formation gradually decrease with incubation time, this may be due to the lack of nutrients, production of toxic metabolites or changes of pH in the culture medium(Data not shown).

Temperature and pH

The optimum temperature and pH for biofilm formation of the isolate were found at 37ºC and 6.5 respectively (Data not shown). The effect of pH values on biofilm formation was found significant. Tang *et al.,*[16] reported that, in case of *S. aureus* the rise or drop in the pH value (≥ 12 or 4 ≤) was directly involved in the decrease of biofilm formation. Our result also suggests that extremely low or high pH value effect on the biofilm formation.

Effect of different Carbon and nitrogen sources

The ability of biofilm formation assay in presence and absence of some dietary carbohydrates showed that more biofilm formation is related to presence of the sugars. Numerous studies have been established the role of sugars in caries etiology and the importance of sugars as the principal dietary substrate that drives the caries process [4,17,18]. In present study (Table 5), highest

biofilm formation was induced in the presence of glucose (conc. @ 1350µg/200µL) by the isolate *S. mutans*. This result are in concurrence with the observation laid down by Christensen *et al*.[19] that bacterial adherence and glycocalyx formation was enhanced with the supplementation of glucose in the culture media. These carbohydrates may induce the expression of some proteins that are responsible for bacterial adherence. Buhler *et al*.[20],demonstrated that *E. coli* biofilm formation increased with increasing glucose concentrations and the results indicate that higher glucose concentrations may be beneficial for biofilm formation, independently of the hydrodynamic conditions in microtiter plates. Although in our study, enhanced biofilm formation was observed with increasing concentrations of glucose in the medium but biofilm formation was then gradually diminished. This indicates that biofilm formation was increased to a certain level of glucose concentration in the medium and the gradual diminished may be an effect of glucose concentration on the microbe. During the investigation of response of biofilm formation of the selected isolate with the utilization of different inorganic and complex organic nitrogenous compounds as nitrogen source, it was found (Table 6) that yeast extract was suitable nitrogen source for biofilm formation (Conc. @1800 µg/200µL). However, Lawrence *et al*.[21], found peptone as the best nitrogen source for biofilm formation by *Pseudomonas fluorescens.* These results suggest that the effect of nitrogenous compound in the formation of biofilm differ from strain to strain.

In vitro evaluation of sensitivity assay

Antibiotic sensitivity assay

The zone of growth inhibition of the selected isolates produced in the presence of antibiotics was shown in table 7. Among the nine antibiotics, penicillin and cefixime did not produce any zone of growth inhibition of the organisms tested here in. These results indicate that these two antibiotics are no longer effective. According to CLSI's guideline, the results of antibiotic sensitivity assay were also interpreted as susceptible, intermediate and resistant. From this study, it was found that *S. mutans* was sensitive to ciprofloxacin and tetracycline; intermediate to chloramphenicol and gentamicin; resistant to amoxicillin, ampicillin, penicillin, cefixime and erythromycin. *S. aureus* ATCC 25923 was found sensitive to ciprofloxacin, erythromycin, chloramphenicol, tetracycline; intermediate to gentamicin; resistant to amoxicillin, ampicillin, penicillin, cefixime. The results indicate gradual development of resistance to antibiotics by the isolate and ciprofloxacin can be considered as suitable alternative oral antibiotic to treat the isolate. High level of resistance to penicillin and amoxicillin and sensitivity to ciprofloxacin by viridians group of streptococci was reported[22]. David *et al.*[23] in one study found that *S. mutans* was 100% resistant to erythromycin. Dautle *et al.*[24] in study found that *Staphylococcus* genus possess the highest diversity for antibiotic resistance to amoxicillin, ampicillin, ciprofloxacin, gentamicin and vancomycin and our results similar with their report.

Determination of MIC and MBC

In this experiment, six different commercially available antibiotics and one mouthwash solution were used to test the MIC and MBC values of those antimicrobial agents against the selected isolates following broth micro-dilution technique. The results of MIC and MBC were shown in table 8 and 9 respectively. The results of MIC were interpreted according to the guideline of CLSI. In this study, it was found that, the MIC values of amoxicillin, penicillin and cefixime fall within the range of resistant category for both isolates and showed sensitivity to tetracycline and ciprofloxacin. In case of erythromycin, *S. mutans* showed resistance while *S. aureus* was sensitive to it. Chlorohexidine gluconate has a much lower MIC value (0.005 µg/mL). It has much cidal effect than tested antibiotics to all of these bacterial isolates. The MBC values give an idea about the actual concentration of antibiotics in *in-vitro* condition to kill 99.9 % of bacteria. In case of some antibiotics, the MBC values were much higher than MBC values.

Table 1
Morphological, cultural and Biochemical characteristics of the selected isolate (KA_{23})

Vegetative cells	: Cocci, single, pair or short chain, 0.5-0.7 µm			
Gram staining	: Gram positive			
Spore staining	: Non spore former			
Acid fast staining	: Non acid fast			
Motility test	: Non motile			
Catalase test	: Negative			
Oxygen relationship	: Facultative anaerobic			
Indole test	: Negative			
Nitrate reduction test	: Negative			
H_2S production	: Negative			
Starch hydrolysis	: Positive			
Gelatin hydrolysis	: Negative			
Methyl red test	: Positive			
Voges-Proskaur (VP) test	: Negative			
Urease test	: Negative			
Oxidase test	: Negative			
Growth at Different temperature	: 10˚C	27˚C	37˚C	45˚C
	-	++	+++	-
Growth at different NaCl (%)	: 0.5	2.5	4.5	6.5
	+++	+	+	-
Fermentation of carbohydrates	: Acid in Glucose, Sucrose, Fructose, lactose, and Manitol; Alkali in Xylose and No fermentation in Arabinose			

Table 2
Screening of the isolates for biofilm formation by test tube assay

Isolates	Visual observation	Absorbance of destained solution	Remarks
Control	-	0.67	No biofilm formation
KA1	-	0.73	No biofilm formation
KA3	+	0.88	Weak biofilm former
KA4	+	0.95	Weak biofilm former
KA7	+ +	1.13	Moderate biofilm former
KA13	+ +	1.63	Moderate biofilm former
KA18	+ +	1.07	Moderate biofilm former
KA22	+ + +	1.80	Strong biofilm former
KA23	+ + +	1.89	Strong biofilm former

Notes: - = No biofilm formation, + = Weak biofilm formation,
++ = Moderate biofilm formation, +++ = Strong biofilm formation

Table 3
Screening of the isolates for biofilm formation by cover slip assay

Isolates	Visual observation	Total pixel area	Mean of dark spots	Mean per pixel area	Remarks
Control	-	8040754	117.464	1.46086E-05	No biofilm formation
KA3	+	4782624	119.651	2.50179E-05	Weak biofilm former
KA4	+	5397796	121.119	2.24386E-05	Weak biofilm former
KA7	+	5065012	118.959	2.34864E-05	Weak biofilm former
KA13	+++	1873476	116.825	6.23574E-05	Strong biofilm former
KA18	+	4160304	118.366	2.84513E-05	Weak biofilm former
KA22	++	2564432	121.187	4.72569E-05	Moderate biofilm former
KA23	+++	2009340	121.008	6.02228E-05	Strong biofilm former

Notes: - = No biofilm formation, + = Weak biofilm formation,
++ = Moderate biofilm formation, +++ = Strong biofilm formation

Table 4
Screening of the isolates for biofilm formation by microtiter plate assay

Isolates	Absorbance of destained solution	Remarks
Control	0.067	No biofilm formation
KA3	0.092	Weak biofilm former
KA4	0.123	Weak biofilm former
KA7	0.092	No biofilm formation
KA13	0.179	Moderate biofilm former
KA18	0.089	No / weak biofilm former
KA22	0.161	Moderate biofilm former
KA23	0.207	Moderate biofilm former

Table 5
The response of biofilm formation of the isolate during the utilization of different carbohydrates as the sole carbon source

Concentration of sugar (µg/200µL)	Absorbance (at 630 nm) of destained solution of biofilms produced by *S. mutans* in presence of different sugars					
	Control	Starch	Glucose	Sucrose	Fructose	Lactose
180	0.078	0.094	0.202	0.192	0.113	0.141
450	0.078	0.135	0.232	0.213	0.132	0.165
900	0.078	0.153	0.247	0.244	0.139	0.104
1350	0.078	0.133	0.254*	0.221	0.159	0.119
1800	0.078	0.126	0.199	0.191	0.148	0.109

Note: '*' Indicates highest absorbance i.e. maximum biofilm formation

Table 6
The response of biofilm formation of the isolate (S. mutans) during the utilization of different nitrogenous compound as the sole nitrogen source

Concentration of nitrogen source (µg/200µL)	Absorbance (at 630 nm) of destained solution						
	Control	Beef extract	Yeast extract	Peptone	Tryptone	KNO_3	NH_4NO_3
180	0.073	0.184	0.189	0.193	0.185	0.196	0.201
450	0.073	0.205	0.219	0.208	0.189	0.202	0.208
900	0.073	0.247	0.259	0.239	0.224	0.189	0.245
1350	0.073	0.201	0.279	0.244	0.259	0.197	0.197
1800	0.073	0.200	0.313*	0.201	0.214	0.195	0.207

Note: '*' Indicates highest absorbance i.e. maximum biofilm formation

Table 7
Showing zone of inhibition of the selected isolates in antibiotic sensitivity assay

Isolates	Zone of inhibition caused by different antibiotics (in mm)								
	AMX	AMP	PEN	CEF	CIP	ERY	GEN	CHL	TET
S. aureus ATCC 25923	25	12	0	0	27	23	13	38	32
S. mutans	0	0	0	0	22	10	14	18	25

Notes: AMP = Ampicillin, AMX = Amoxicillin, CEF = Cefixime, CHL = Chloramphenicol, CIP = Ciprofloxacin, ERY = Erythromycin, GEN = Gentamicin, PEN = Penicillin, TET = Tetracycline

Table 8
The MIC of different antibiotics & mouth wash solution against the selected bacterial isolates

Isolates	Minimum Inhibitory Concentration (µg/mL)						
	AMX	TET	PEN	CEF	CIP	ERY	CHX
S. aureus ATCC 25923	2	4	128	8	1	2	0.005
S. mutans	32	2	>256	8	1	2	0.005

Note: CHX = Chlorohexidine gluconate

Table 8
The MBC of different antibiotics and mouthwash solution against the selected bacterial isolates

Isolates	Minimum Bactericidal Concentration (µg/mL)						
	AMX	TET	PEN	CEF	CIP	ERY	CHX
S. aureus ATCC 25923	4	16	>256	64	4	4	0.005
S. mutans	>256	8	>256	32	4	8	0.032

CONCLUSION

As dental caries is a major public health problem and current clinical preventive approaches have a minimal impact on addressing oral health inequalities. Strategic and coordinated public health action is needed to tackle the main cause of dental caries. Our studies showed that dietary sugars have significant impact on biofilm formation of *S. mutans*. Therefore, our food policy should reduce the excessive consumption of sugary foods and drinks. Antibiotic resistance is another important concern now a day, so sample culture and antibiotic sensitivity test should be routinely performed in all patients. Depending on the antibiotic susceptibility, therapy should be designed of an antibacterial agent with the narrowest spectrum, least cost and little adverse effect. If development of antibiotic resistance is going uncontrollable, time is not so far, when an effective antibiotic would not be able to treat even minor infection. This study showed high prevalence of multidrug resistant bacteria at Dental unit in Chittagong Medical College, Bangladesh. Further study on molecular screening and genomic analysis may be done for identifying specific genes related to antimicrobial resistance.

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