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In Vitro Biofilm Formation Potential and Antimicrobial Sensitivity of *Streptococcus mutans* Clinical Isolates

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ABSTRACT

Dental caries is the major public health problem that disturbs all countries in the world. *Streptococcus mutans* is considered as a chief culprit of this infectious disease and biofilm formation potential is the major virulence trait of this pathogen. In the era of antimicrobial resistance it is important to understand the virulence mechanism and antimicrobial sensitivity of *S. mutans* globally. The objective of the study was to investigate the biofilm formation potential and antimicrobial sensitivity of clinical isolates of *S. mutans*. Biofilm formation potential of 100 clinical isolates was studied by microtiter plate assay and coverslip assay and antimicrobial sensitivity was assessed by disc diffusion method. Our results showed that out of 100 clinical isolates 92 (92%) showed strong biofilm forming capability and 8 (8%) clinical isolates showed moderate biofilm formation potential. Antimicrobial sensitivity results showed that 72 (72%) isolates were resistant to amoxicillin, 65 (65%) isolates were resistant to chloramphenicol, 40 (40%) isolates were resistant to doxicycline, 46 (46%) isolates were resistant to erythromycin, 32 (32%) isolates were resistant to ofloxacin, 61 (61%) isolates were resistant to tetracycline and only 17 (17%) isolates of *S. mutans* were resistant to amoxicillin/clavulanate. Our results suggest that combination therapy is more effective against cariogenic *S. mutans* and biofilm formation potential of this organism indicates the powerful contribution in pathogenesis. Ultimately, new combination therapies and inhibitors of biofilm formation are urgently needed.

Key words: antimicrobial, biofilm, dental caries, *S. mutans*, therapy, virulence

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INTRODUCTION:

Oral infections related to dental biofilm continue to afflict the majority of the world's human population. Among them, dental caries continues to be the single most prevalent and costly oral infectious disease. Almost fifty years ago it was recognized that *Streptococcus mutans* played an important role in cariogenesis. Biochemical approaches identified three important unique properties of these organisms which appear to be important in disease initiation (acid uricity, acidogenesis and sucrose dependent colonization). Much attention has been focused on the mechanism by which *S. mutans* colonizes tooth surfaces. Both biochemical and molecular approaches suggest that this occurs via a two-step process: a sugar independent attachment to the tooth pellicle modulated by *S. mutans* adhesions, ionic or hydrophobic interactions, as well as impaction of the cervices on the tooth surface followed by a sucrose-dependent enhancement of tooth colonization. *S. mutans* effectively utilizes dietary sucrose to synthesize large amounts of exo polysachharides through invertase, glucosyl transferases (Gtfs) and fructosyl tranferases (Ftfs). The virulence determinants of *S. mutans* have received much more attention over several decades and have been investigated using a range of biochemical, physiological and genetic techniques. The most extensively studied virulence determinants are Gtfs, which play a role in adhesion, contributing to the production of extracellular polysaccharides (Figure 1), that localizes both the bacteria and their secreted products within the plaque matrix[1].

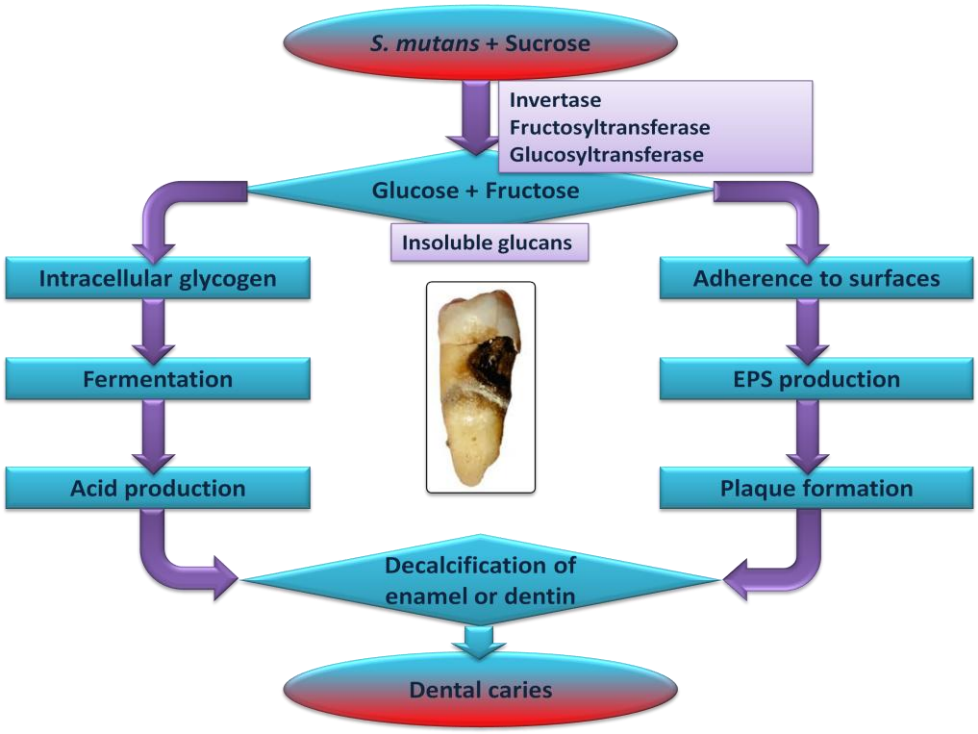

Figure 1 Sucrose utilization and pathogenesis of dental caries by *S. mutans*.

S. mutans cells attached to teeth as part of a biofilm (dental plaque) may exhibit a distinct mode of growth which differs from that of planktonic cells, and this distinct mode of growth can be characterized by an increased resistance to antibiotics as wells by differential gene expression[2]. In the present clinical scenario globally, there is a great interest in the use of antimicrobial agents for prevention and treatment of dental caries due to the spread of antibiotic resistance. The widespread concern about the increasing problem of antibiotic resistance has emphasized the need for rationalization of antibiotic use in the treatment of dental caries[3]. Inappropriate antibiotic prescribing and use have been identified as major factors in the emergence of antibiotic resistance in *S. mutans*. Consequently, modification and surveillance of prescribing attitudes have become crucial[4,5]. The present study focused to optimize biofilm formation potential and antimicrobial sensitivity pattern of *S. mutans*.

MATERIALS AND METHODS

Bacterial isolation and identification

A total of 142 *Streptococci* were isolated from dental caries or plaque (normal teeth, without any periodontal disease) of the 30 patients of People's dental academy, Bhopal, (MP), India. These patients were of both sexes with the mean age of 20 years. The standard strain was *S. mutans* ATCC 25175. All strains were cultured on the media such as Brain Heart Infusion agar (BHI, Himedia Laboratories, India) in a 5% CO_2 enriched atmosphere. The biochemical tests were done for their identification[6]. Among 142 *Streptococci*, 100 isolates were identified as *S. mutans.* The study was approved by the ethical committee (PDA ETH.-11/001) of People's Dental Academy, Bhopal (MP).

Biofilm formation by Microtiter plate assay and coverslip method

Quantification of biofilm formation of *S. mutans* isolates was done by using microtiter plate method. To assay biofilm formation of the *S. mutans* isolates, an overnight culture of each was grown in brain heart infusion broth (Himedia Laboratories, India) for 18-20 hours at 37°C. One ml of each overnight culture was transferred to 10 ml of sterile BHI broth with the addition of 1% Sucrose for production of biofilm. The suspensions were adjusted with the same BHI medium to 0.5 on the McFarland turbidity standard as measured by absorbance (0.08 - 0.1 at 625 nm) in a spectrophotometer (Shimadzu, Australia), corresponding to approximately 102 CFU/ml. Then, from each culture, 250 µl volumes were transferred into wells of a microtiter plate **(**Himedia Laboratories, India**)**[7]**.** Blank wells contained broth, only. Plates were made in triplicate and incubated for 24 hours at 37°C. At the end of 24 hours, the planktonic suspension and

nutrient solution were aspirated and each well was washed three times with 300 µl of sterile physiological saline. The plates were strongly shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 250 µl of 96% ethanol per well and, after 15 min, plates were made empty and left to dry. Each well was then stained for 5 min with 200 µl of 2% crystal violet (CV Gram stain, Merck, Germany). The stain was rinsed off by placing the plates under running tap water. After drying the stained plates, biofilms were visible as purple rings formed on the sides of each well. The quantitative analysis of biofilm formation was performed by adding 200 µl of 33% (v/v) glacial acetic acid (Merck, Germany) per well. Then the optical density (OD) of the stain was measured at 492 nm by an ELISA reader (Lisa, Germany) as described previously[7]. Biofilm formation was scored as follows: –, non-biofilm-forming ($A_{492} \leq 1$); +, weak ($1 \leq A_{492} \leq 2$); ++, moderate ($2 < A_{492} \leq 3$); +++, strong ($A_{492} > 3$). Microtiter assay was performed in triplicate.

In coverslip method, biofilm of *S. mutans* clinical isolates were grown as follows, individual sterile culture dishes were filled with 2.5 ml of BHI broth with 1% sucrose and sterile 18 mm diameter glass microscope coverslip was added to each dish, and culture dish was covered. Each sample was inoculated with defined volume of overnight culture. The dishes were incubated micro aerobically at 37ºC for 48 hr. Glass cover slips containing attached biofilm were removed from dishes and rinsed briefly with PBS and stained with 0.5% crystal violet for 5 min. Stained biofilm were observed microscopically[8].

Antimicrobial Sensitivity

The antimicrobial sensitivity of the test strains to different antibiotics i.e., amoxicillin (30mcg), amoxicillin/clavulanate (30mcg), chloramphenicol (30mcg), doxycycline (30mcg), erythromycin (15mcg), ofloxacin (5mcg), and tetracycline (30mcg) was determined by disc diffusion method[9]. A lawn of test pathogen was prepared by evenly spreading 100 µl inoculums (1.5×10^8 CFU/ml) on BHI agar plates. The plates were allowed to dry before applying antibiotic discs. The discs were firmly applied to the surface of agar plates within 15 minutes of inoculation. The contact of the discs to agar was even. The agar plates were incubated at 37ºC for 24 hours. If antimicrobial activity was present on the plates, it was indicated by an inhibition zones in the millimeter at 24 hours were measured using a zone scale (Himedia Laboratories, India). The experiments were conducted in triplicate. For each antibiotic, the mean and standard deviation of the diameter of inhibition zones was calculated. Antibiotic susceptibility was established and measured by the reference criteria of Clinical and Laboratory Standards Institute (CLSI) guidelines[10].

RESULTS AND DISCUSSION

Among 100 isolates of *S. mutans*, 92 (92%) isolates showed strong biofilm activity and only 8 (8%) isolates showed moderate biofilm formation capability. Strong biofilm forming capability of *S. mutans* on coverslip was also observed microscopically (Figure 2). Our results showed that the biofilm formation potential varied from strain to strain. The strongly adhesion of *S. mutans* clinical isolates to abiotic surfaces indicate the major role in their pathogenicity in oral cavity. Antimicrobial sensitivity results showed that among 100 isolates of *S. mutans*, 72 (72%) isolates were amoxicillin resistant, 65 (65%) isolates were chloramphenicol resistant, 40 (40%) isolates were doxicycline resistant, 46 (46%) isolates were erythromycin resistant, 32 (32%) isolates were ofloxacin resistant, 61 (61%) isolates were tetracycline resistant and only 17 (17%) isolates were resistant against amoxicillin/clavulanate. Amoxicillin/clavulanate is more effective against cariogenic *S. mutans* among other drug tested (Figure 3). Our results from the present investigation substantiate the use of combination therapy in dental practice. It may be suggested that due to lack of appropriate knowledge of prescribing antibiotics for the treatment of dental caries on part of dental professionals, the microbial flora responsible for causing dental caries has developed resistance to the antimicrobials. The strong biofilm formation capability and antibiotic resistant isolates indicate the powerful contribution in the pathogenesis of dental caries.

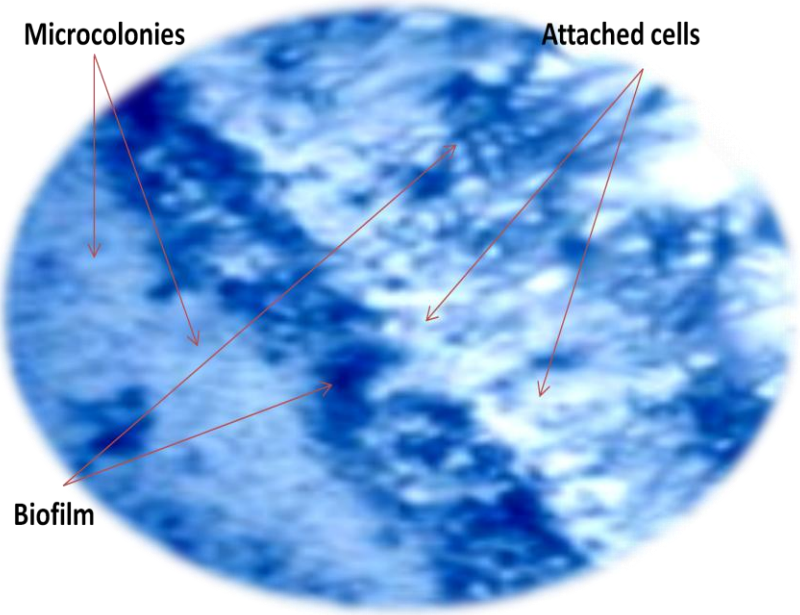

Figure 2 Microscopic view of biofilm development by *S. mutans*.

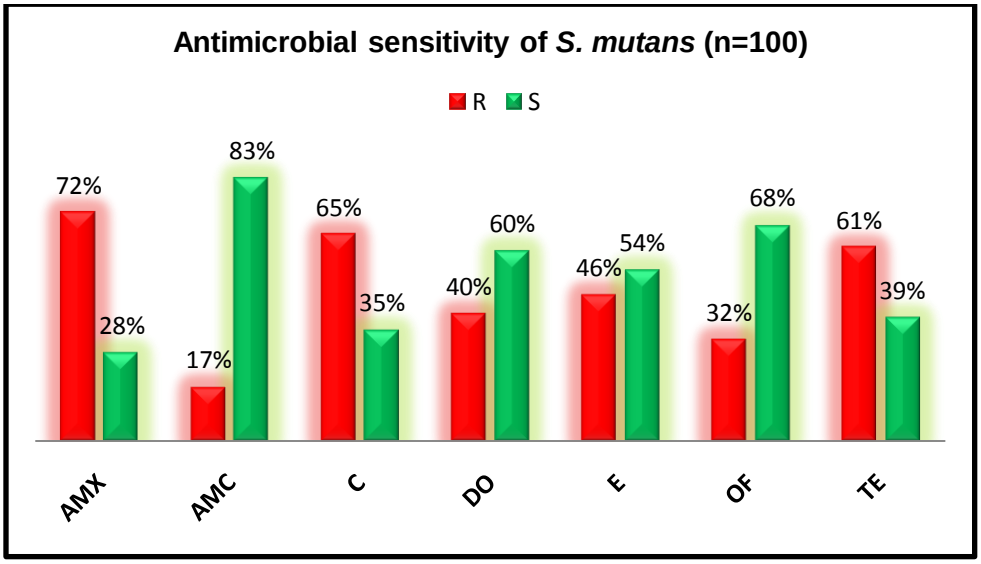

Figure 3 Distribution of antimicrobial sensitivity of *S. mutans* clinical isolates. R = Resistant, S = Sensitive, Amoxicillin (AMX), Amoxicillin/clavulanate (AMC), Chloramphenicol (C), Doxycycline (DO), Erythromycin (E), Ofloxacin (OF) and Tetracycline (TE).

CONCLUSION

We concluded that the biofilm formation by *S. mutans* clinical isolates plays major role in the pathogenicity and may also contribute in the antimicrobial resistance. Still combination therapy is effective against *S. mutans.* Furthermore, biofilm control strategies based on disruption of exo polysaccharide and development of quoram sensing inhibitors offer an attractive and alternative approach in the field of chemotherapy for dental caries.

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