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PHENOTYPIC AND MOLECULAR IDENTIFICATION OF NON-MUTANS STREPTOCOCCI ORGANISMS RECOVERED ON MITIS-SALIVARIUS BACITRACIN AGAR FROM CARIES ACTIVE SUBJECTS

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ABSTRACT

Dental caries is one of the most common oral infectious disease affecting humans worldwide and Mutans Streptococci (MS) have been implicated as major cariogenic bacteria. The "low-pH non-Mutans Streptococci Organisms (non-MSO) have been identified as atypical strains in caries plaque samples, in the absence of MS group. The non-MSO usually competes with MS group for colonizing on the tooth surface. The objective of the present study was to isolate and identify the non-MSO recovered on Mitis-Salivarius Bacitracin (MSB) agar from caries active subjects. Dental plaque samples were collected from caries active subjects and cultured on MSB agar. The bacteria grown on MSB agar were subjected to biochemical tests and 16S rDNA identification. Among 38 subjects, 4 subjects were positive for non-MSO, which were identified as *Streptococcus anginosus* (03) and *Streptococcus sanguinis* (01). The detection of non-MSO in caries active subjects, confirmed their involvement in the infection which may support "Non-specific plaque hypothesis" of caries formation.

KEY WORDS: Dental caries, Mitis-Salivarius Bacitracin Agar, Biochemical test, Non-mutans streptococci organism, 16S rDNA

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INTRODUCTION

Dental caries is a ubiquitous and oldest prevailing disease affecting all age groups and segments of mankind [1, 2]. There are three main schools of thought on the role of plaque bacteria in the etiology of caries (i) the specific plaque hypothesis, (ii) the nonspecific plaque hypothesis, and (iii) the ecological plaque hypothesis [3, 4, 5, 6]. The specific plaque hypothesis proposed that only a few specific species comprising the resident plaque microflora are actively involved in the disease [3, 5]. In contrast, the "Non-Specific Plaque Hypothesis" which maintains that caries results due to the overall activity of total plaque microflora and heterogeneous group of microbes play the role in the disease [6, 7]. The ecological plaque hypothesis states that there is a shift in the balance of cariogenic bacteria from neutral pH to low pH due to frequency of consumption of fermentable carbohydrates resulting in enrichment of acidogenic and aciduric species in plaque [8]. The oral streptococci are classified into four species groups namely the anginosus, mitis, mutans and salivarius [9]. The classification is based on chemotaxonomic and genotypic data, especially DNA-DNA base pairing and 16S rRNA gene sequence analysis. Among the oral streptococci group, Mutans Streptococci (MS) are constantly associated with dental caries which comprises of seven species namely *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus downei*, *Streptococcus cricetus*, *Streptococcus rattus*, *Streptococcus ferus*, and *Streptococcus macacae* [10, 11, 12]. *S. mutans* and *S. sobrinus* are strongly associated as they are frequently isolated from caries subjects [13, 14]. MS have received extensive attention as etiological agents responsible for dental caries, though they don't always constitute the major proportion of microflora in primary carious lesions and only increase in their number as the caries progress [15]. The pioneering work of Clarke [16] revealed that the caries sometimes develops in the absence of any detectable *S. mutans*. These findings suggest that the other oral species might produce similar amounts of acids which cause the disease. Few investigators have reported that it is probably non-Mutans Streptococci Organisms (non-MSO) and *Actinomyces* that contribute in the early stages of acidification, demineralization of enamel which results in aiding the MS and other oral bacteria to move in for capitalize the environment [17]. Mitis-Salivarius agar is commonly used for selective isolation of oral streptococci from plaque samples [18, 19] and was modified to MSB agar by Gold *et al.*, [20] for the recovery of *S. mutans* with maximum inhibition of the balance of the streptococcal flora normally encountered on the Mitis-Salivarius agar. Yoo *et al.,*[21] were probably first to report the species level identification of non-MSO recovered on MSB agar and highlighted the scarcity of information with reference to non-MSO. These investigators have also outlined the importance of identification of non-MSO on MSB agar which will help in improving this medium. The study to investigate the dynamics of non-MSO in caries subjects is important for treatment, prevention strategies and in developing anti-caries agents focused towards mixed microbial species. In this background, our investigation was carried out with the following objective to isolate and identify the non-MSO recovered on MSB agar from caries active subjects.

MATERIALS AND METHODS

STUDY POPULATION

The present study was approved by the institutional ethical committee at M.S. Ramaiah Dental College, affiliated to Rajiv Gandhi University of Health Sciences, Bangalore. The study comprised of 38 cases, which includes 19 males and 19 females respectively, ranging in the age from 35 to 44 years as per the WHO guidelines [22]. The nature of the work followed in the present study was fully explained to all participants and the study was conducted with formal written informed consent. The subjects were screened using a pathfinder survey and who volunteered in the study was interviewed using a questionnaire. Qualified subjects had no chronic disease or had not received antibiotic therapy for atleast 6 weeks [23]. The clinical examination was conducted in duplicate by calibrated dentist to evaluate intra-examiner reliability.

DENTAL PLAQUE COLLECTION

Sterile tongue depressor was used to avoid contamination from other mouth parts and to aid a better vision of carious lesions. Plaque sampling sites varied depending on the condition of the oral cavity. The plaque samples were collected from carious lesions sites with sterile wooden toothpicks. The tips of the toothpicks were aseptically cutoff and immediately transferred into 1ml of sterile phosphate buffer saline and stored at 4ºC [24].

BACTERIAL ISOLATION FROM DENTAL PLAQUES

The Mitis-Salivarius agar (Himedia) was modified to MSB agar (Gold *et al*.,) [20] by adding 20% sucrose (Himedia) and 0.2 units/ml bacitracin (Himedia).The samples were vortexed and plated on MSB agar followed by anaerobic incubation for 37ºC at 48h.

MORPHOLOGICAL IDENTIFICATION

After incubation period, the colonies of MS and non-MSO were selected based on colony morphology [12, 24]. From each sample plate, suspected colonies of MS and non-MSO were picked up and transferred to 2 ml of Brain Heart Infusion (BHI) broth (Himedia) and incubated at 37ºC for 18h. The Gram's nature and morphology of the bacteria were determined by Gram's staining.

BIOTYPING

Colonies were further confirmed by biochemical tests for fermentation of mannitol, sorbitol, melibiose, raffinose and hydrolysis of arginine [25, 26]. Sterile carbohydrate discs (Himedia) were aspectially added to phenol red broth base (Himedia) which was used as a basal medium for the fermentation of carbohydrates and for arginine hydrolysis, arginine dihydrolase broth (Himedia) was used. The tubes were inoculated with overnight culture of test organisms and incubated at 37°C for 48h. A positive reaction for fermentation was indicated by color change from red to yellow while arginine hydrolysis was deducted by the color change from purple to yellow and then back to purple. The biochemical results were validated with reference strains and repeated in order to confirm reproducibility and reliability. The bacteria which were remained unidentified by biochemical scheme proposed by Shklair and Keene [25, 26] were considered as non-MSO. After identification, the cultures were maintained at -20°C in 10% glycerol BHI broth (Himedia).

DNA EXTRACTION AND PURIFICATION

DNA was extracted and purified according to Spolidorio *et al.,* [27] and Bert *et al.,* [28] with some modifications. Single colony forming units of each strain were inoculated in BHI broth and incubated at 37ºC for 18h. The cells were pelleted down and washed twice with TE buffer (50 mM Tris, 1 mM EDTA, pH 8)[Sigma Aldrich], centrifuged and resuspended in buffer containing 10 mM Tris-HCl, 50 mM EDTA (Sigma Aldrich), and 25% sucrose (Himedia). DNA was extracted from bacterial cells by incubation with 10 mg/ml lysozyme (Himedia) and 100 µg/ml RNAase (Sigma Aldrich) for 30 minutes at 60ºC followed by incubation with 50µl of 10 mg/ml proteinase K (Himedia) and 50µl of 10% sarkosyl (Sigma Aldrich) at 37ºC for 2h and then for 30 min at 68ºC. After enzymatic treatment, the pellet was treated twice with phenol: chloroform: isoamyl alcohol (25: 24:1) [Qualigens]. DNA was precipitated with double volume of ice cold ethanol (Himedia) and stored at -20ºC for 30 minutes. The vials were centrifuged and the pellet was washed with 70% alcohol. The pellet was air dried and dissolved in TE buffer and stored at -20ºC until use. The DNA was further purified using column purification kit (Qiagen). Concentrations of eluted DNA samples were determined by measuring A260nm using UV spectrophotometer (UV-1800 Shimadzu). The purity of DNA was evaluated from A260/A280 ratio [29]. The isolated DNA was electrophoretically checked by loading DNA sample with loading dye on 0.8% agarose gel.

16S rDNA PCR AMPLIFICATION AND IDENTIFICATION

PCR amplification for 16S rDNA gene was done with primers F- 5´AGT TGA TCC TGG CTC AG 3´ and R- 5´ACC TTG TTA CGA CTT 3´. The PCR (PerkinElmer 2009) conditions were initial denaturation at 94ºC for 2 minutes followed by denaturation at 94ºC for 30 seconds. Annealing at 58ºC for 30 seconds and extension at 72ºC for 90 seconds and

final extension for 72ºC for 2minutes. This amplification was repeated for 30 cycles. The PCR product was run in 1% agarose gel, 1.5 Kb band was then purified with Gel elution Kit, ARK-07(Aristogene). The product was subjected to 16S rDNA sequencing (ABI 3730xl 96 well capillary sequencer, Applied Biosystem). The resultant sequences were subjected to BLAST search in relevant database and the species were identified. The sequences were submitted to NCBI database to obtain GenBank accession numbers.

RESULTS

Among the 38 clinical isolates from caries active subjects, 4(10.52%) of them were identified as non-MSO by 16S rDNA sequencing. Of these four isolates, three were *S. anginosus* and one was *S. sanguinis*. The four non-MSO showed typical MS colony morphology (mulberry shaped) on MSB agar. Fig1. presents the proportion of non-MSO (10.52%) and MS(89.47%) recovered on MSB agar. The bacteria, which were remained unidentified by biochemical scheme is presented in table 1.

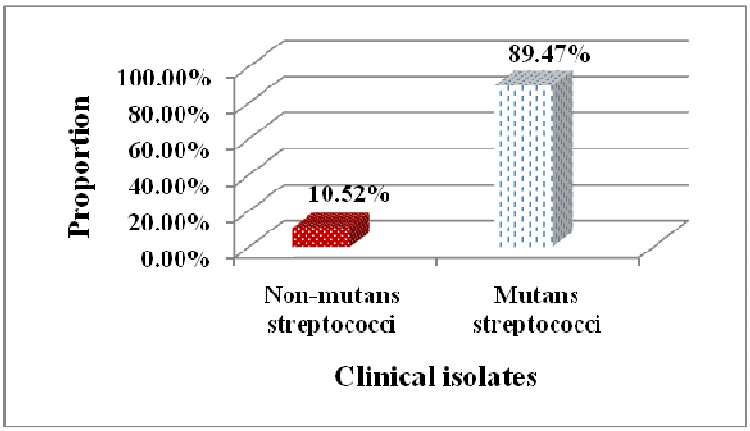

Figure 1
The proportion of non-MSO and MS recovered on MSB agar.

Table 1
Biochemical tests results of non-MSO isolated from plaque samples.

Strain	Mannitol	Sorbitol	Raffinose	Melibiose	Arginine	Mannitol + 2U/ml Bacitracin
P3	-	-	-	-	+	-
P4	-	-	+	-	+	-
P8	-	-	-	-	+	-
P33	-	-	+	+	+	-

The GenBank accesssion numbers provided by NCBI and identification of non-MSO isolates at species level by 16S rDNA sequencing is presented in table 2.

Table 2
GenBank accesssion numbers and identification of non-MSO isolates in the study population

Strain	GenBank Accession Number	16S rDNA sequencing identification
P3	JX678670	*S. anginosus*
P4	JX678671	*S. anginosus*
P8	JX678675	*S. anginosus*
P33	JX678700	*S. sanguinis*

DISCUSSION

Among 38 clinical isolates from caries active subjects, 4(10.52%) were detected to be non-MSO, these findings are in accordance to Russell [17]. The non-MSO was unable to be differentiated from MS based on colony morphology. The results from the present study indicate that the MSB agar could not comprehensively inhibit non-MSO and was inadequate to differentiate the oral streptococci species. Our study is in agreement with Russell [17] in making a Type II error, failing to detect the true bacteria, based on morphology. The biotyping results showed that there were different phenotypes of *S. anginosus* in study population and similar result was reported by Yoo *et al.,*[21]. The possible explanation for detecting *S. sanguinis* is that they usually compete with the MS group for colonizing on the surface of the tooth [30]. In addition to that Takahashi [15] has reported, MS are inhibited by hypothiocyanite which is produced by the metabolic activity of *S. sanguinis.* Yamaguchi *et al.,* [31] has reported that *S. sanguinis*, has low cariogenicity and aids in aggregation of oral bacteria and maturation of dental plaque. A previous study has reported that *S. anginosus* were able to grow on MS-SOB medium which contains multiple antibiotic namely Bacitracin, Aztreonam and Fosfomycin [32] and in an another study Yoo *et al.,* [21] reported 8 strains of *S. anginosus* and one strain of *S. sanguinis* were able to grow on MSB agar. These findings clearly indicate that both species have gained resistance over time. There are sufficient studies on *S. anginosus* involved in carcinogenesis, [33, 34] but there is a scarcity of information on *S. anginosus* involvement in caries. Our study indicates that both *S. sanguinis* and *S. anginosus* have acquired resistance to both 20% sucrose and 0.2 units/ml bacitracin, and was able to grow on MSB agar in accordance previous findings [21].

CONCLUSION

The study concludes by stating that MSB agar does not suppress sufficient non-MSO. Presence of non-MSO in the caries active subjects confirmed their involvement in caries infection which may support “Non-specific plaque hypothesis” of caries formation. The non-MSO usually not preferring MSB agar might have gained antibiotic resistance and behaved like MS. This behavior of non-MSO might have caused difficulty in identifying them on MSB agar but warranted molecular characterization. All the four isolates were identified as non-MSO by 16S rDNA sequencing study.

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