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ORIGINAL ARTICLE

DNA finger printing of *S. Mutans* present in the saliva of caries active children and those associated with intellectual disability – An RAPD analysis

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Abstract *Aim:* The aim of this study is, to evaluate and compare the diversity of *S. Mutans* genotypes with respect to caries activity among normal children and intellectually disabled children, which would enable the clinician to plan better strategies for early caries detection, management and prevention.

Materials and methods: Genotyping of *S. Mutans* was done by collecting the saliva samples from 40 caries active children (20 normal and 20 children associated with intellectual disability by Rapid amplified polymorphic DNA analysis using three arbitrarily primers (P1, P2, P3). Rapid amplified polymorphic DNA (RAPD) is preferred because of its reliability, reproducibility in generating genetic fingerprints of *Streptococcus* isolates.

Results: Number of bacterial counts in Group I showed a mean of 111.6500 followed by the Group II with a mean of 102.6500. Therefore, the difference in the number of bacterial counts was not significant between the two groups ($p < 0.001$). Genotype encoding Primer 1 was present in almost 82.5% of the total population of both groups. Genotype encoding Primer 2 was present in 95% of the total population. Whereas, Genotype encoding Primer 3 was present in 20% of children associated with intellectual disability and 95% of normal children.

Interpretation and conclusion: There was no significant difference in *S. Mutans* count of normal caries active children to that of caries active children with intellectual disability, but, there was a significance difference in the distribution of *S. Mutans* genotypes in both the groups.

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1. Introduction

Dental caries, otherwise known as tooth decay, is one of the most ubiquitous chronic diseases of people worldwide; individuals are vulnerable to this disease throughout their lifetime. This disease develops as an aggressive tooth decay that effects

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the primary dentition during early childhood (Selwitz et al., 2007).

Oral cavity harbors, a wide range of microbial flora due to its ideal humidity and temperature and the frequent passage of nutrients (Loesche, 1986).

A lesion is said to be active when the following criteria were met:

(1) The Enamel surface manifest a whitish opaque appearance,
(2) It has developed cavitation and
(3) Bitewing radiographs showed the progress of demineralization within the preceding 2 years of period (Nyvad and Kilian, 1990).

The microbial community of caries is diverse and contains many facultative and obligatory-anaerobic bacteria (Tanzer et al., 2001). Among all these, *Streptococcus Mutans* is generally considered as a principal aetiologic agent for dental caries (Loesche, 1986). Decades of research have conclusively demonstrated that *S. Mutans* are a major cariogenic organism by virtue of its contribution to the formation of the dental biofilm matrix, its capacity to produce large quantities of organic acids, and its propensity to outcompete non-cariogenic commensal species at low pH conditions (Banas et al., 2003). According to the official Guidelines for Human Gene Nomenclature, a gene is defined as a DNA segment that contributes to phenotype/function (Gerstein et al., 2007). Genotype is the genetic constitution of a cell, an individual or an organism. There are four serotypes of *S. Mutans* i.e. c, e, f and k of which genotype c is most commonly present in children with dental caries (Arévalo-Ruano et al., 2014). The pathogenic potential of *S mutants* varies with its genotype due to difference in activity of glucosyltransferase among the various genotypes which is known to affect the virulence of s mutants. Children usually carry fewer genotypes than adults i.e. 8–11 (Kyounga et al., 2013).

Studies say that there are more diversified *S. Mutans* genotypes in the group of children with dental caries than the caries-free group and also there exist genotypic variability within population. Genotyping is the process of detecting the genotype of an individual with a biological assay. Also known as genotypic assay, techniques include PCR, DNA fragment analysis, allele-specific oligonucleotide (ASO) probes, DNA sequencing, and nucleic acid hybridization to DNA microarrays or beads (Braker et al., 2001). Intellectual disability (ID) is a serious and lifelong condition that places heavy demands on society and the health system (Roeleveld and Zielhuis, 1997). The World Health Organization (WHO) estimates that globally over 450 million people suffer from psychological disorders. Currently, cognitive and behavioral disorders account for 12% of the global burden of disease (Reddy et al., 2013). The intellectually disabled children usually have associated medical problems in addition to their primary condition and any oral or dental problems may further compromise their general health. Cognitive disability is an essential factor for harboring dental plaque initiating dental caries especially when there is bad oral hygiene and improper diet intake (Faten, 2012).

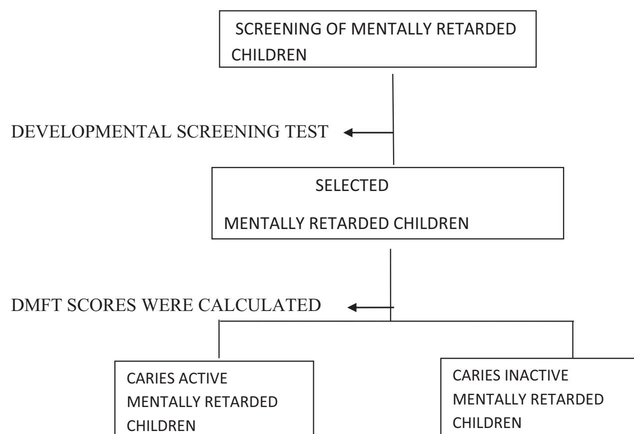
There have been many reports on dental caries in Down syndrome which is one among the cognitive disabilities (Barnett et al., 1986) studies found that *S. Mutans* profiles were present in Down syndrome individuals that were responsible for a low caries rate (Cogulu, 2006).

But there is a paucity of evidence on the genotypic diversity of *S. mutans* in the intellectually disabled children. The possible therapeutic approaches from conventional to the most advanced techniques, which includes, caries vaccine, can only be targeted on caries, only if we have adequate knowledge of its activity. Thus, the aim of this study is to gain an insight into the diversity of *Streptococcus Mutans* genotypes associated with the caries-active normal and intellectually disabled children.

2. Materials and methods

2.1. Materials

Subjects studied were 40 Indian children i.e. 20 caries active children and 20 caries active children with intellectual disability, with an age range of 6–14 years. The present study was conducted in a residential school. The study was approved by the Ethical Committee (Reg.no BDC/EXAM/283/2016-17) and informed consents were obtained from all the children's parents, who children were participating in this study. No previous brushing instructions and dietary modifications was advised to children. A general examination of children was done prior to the study, to assess if he/she comes under selection criteria. The developmental screening test (Dhanesh and Karthikeyan, 2012) was conducted for determining the intelligent quotient level of special children as, some of them were not cooperating for saliva collection. The samples were divided into mild, moderate and severe. Children associated with cognitive disability with IQ (intelligent quotient) level between 58 and 67 (mild) were included in this study.



Selection criteria of caries active Mentally retarded children

2.2. Dental examination

All the selected children were examined while sitting on a chair under natural light. A visual diagnosis was made using a mouth mirror and cotton rolls to assist visibility and a periodontal probe was used to remove any plaque or debris when necessary. Caries experience was measured by the dmft (decayed, missing, and filled teeth) index given by World

Health Organization (WHO). Children between 6 and 14 years of age, Mentally retarded children with IQ level between 58 and 67 and children with dfmt 4 or more (caries active) were included in this study. Children who were non co-operative, with any other concomitant diseases or syndromes and children on antibiotic therapy within 3 months were excluded from the study

Group – 1	Caries active mentally retarded children	Sample size – 20
Group – 2	Caries active Normal children	Sample size – 20

2.3. Microbiological study

Microbiological Media:

- Mitis-Salivarius agar supplemented with 20% sucrose and antibiotic solution.
- Nutrient broth

Primers:

Primers were designed and synthesized for the amplification of template DNA

Primer	5′–3′	GC (%)
P1	CAGGCCCTTTC	70
P2	TGCCGAGCTG	70
P3	CCCGTCAGCA	70

All saliva samples were stored in an ice box and were transported to the laboratory to be processed within two hours of collection. Samples were gently shaken and diluted in tenfold step with repeated homogenization on a vortex mixer for 10 s between successive dilutions. After dilution, 0.1 ml of diluted sample was streaked on Mitis-Salivarius agar plates and incubated at 37 °C for 48 h in an atmosphere of 10% CO2. Detected *S. Mutans* were observed through microscope and the number of colony forming units were counted using digital colony counter (Nanda, 2015) *Streptococcus Mutans* were identified as round or highly convex colonies that were dark blue to black, from pin point to pin head size with a rough surface. Each single spore of *S. Mutans* isolate was grown for 6 days at 28 °C in 5 ml nutrient broth in 15 ml falcon tubes and grown culture was used for DNA isolation.

2.4. Genomic DNA extraction

DNA was extracted from each single–spore isolate by PCR with previously described primers (Oho et al., 2000).

2.5. RAPD using arbitrary primers:

DNA amplification for screening of final genotyping was carried out using 0.12 gm of Tris and 0.0372 gm of EDTA(PH-8.0). Grown 18 h old culture was transferred into sterile Eppendorf tubes and centrifuged at 6000 rpm for 10 min. The temperature cycling program used with a thermocycler (BIO-RAD, 7100) is as follows: 2 initial cycles consisting of 94 °C for 4 min, 35 °C for 2 min, and 72 °C for 2 min, followed by 35 cycles consisting of 94 °C for 30 s, 35 °C for 1 min, and 72 °C for 2 min and a final extension step consisting of 72 °C for 5 min. After electrophoresis on an agarose 1.5%, the gel was observed under the "Gel – documentation system" and Gel images were snapped (Figs. 1 and 2). Individual AP-PCR amplicons were marked and individual bands were visually compared. Finally, documentation was done and photographs were taken. A Dendrogram was constructed using UPGMA method with the aid of NTSYS (numerical and multivariate analysis system program) (Williams et al., 1990).

3. Results

The present molecular study was conducted to evaluate the *S. Mutans* profiles in dental caries. All results were recorded, tabulated & subjected to appropriate statistical analysis using the Statistical Package for the Social Sciences (SPSS Version. 16). Students unpaired 't' test was used for the comparison of *S. Mutans* count.

3.1. S. Mutans count

The mean value of *S. Mutans* count in caries active normal and intellectually disabled children was 111.6500 and the standard deviation was 21.04700.

The mean value of *S. Mutans* count in caries active intellectually disabled children was 102.6500 and the standard deviation was 29.80864.

The 'p' value of both the groups was 0.278 which shows that there was no significant difference of *S. Mutans* in between both the groups (Table 1).

3.2. Genotypic distribution of S. Mutans in between groups

With respect to the distribution of different genotypes (Table 2), children with intellectual disability colonized with *S. Mutans* genotype RAPD pattern coding Primer 1 was 80% ie.,16 children, whereas in caries active normal children it was 85% i.e., 17 children.

With regard to Primer 2, it was present in 90% in caries active children with intellectual disability where as it was 100% in caries active normal children and then Primer 3 was present in 20% of caries active intellectually disabled children and 95% in caries active normal children.

3.3. Genotypic distribution of S. Mutans in overall population

On overall population, 85% of children i.e., both caries active normal and intellectually disabled children had amplification pattern encoded by Primer 1 colonized in the oral cavity (Table 3).

S. Mutans genotype pattern coding the Primer 2 (P2) was present in 95% of intellectually disabled and normal children. And finally *S. Mutans* genotype pattern coding the Primer 3 was present in 57% of the children.

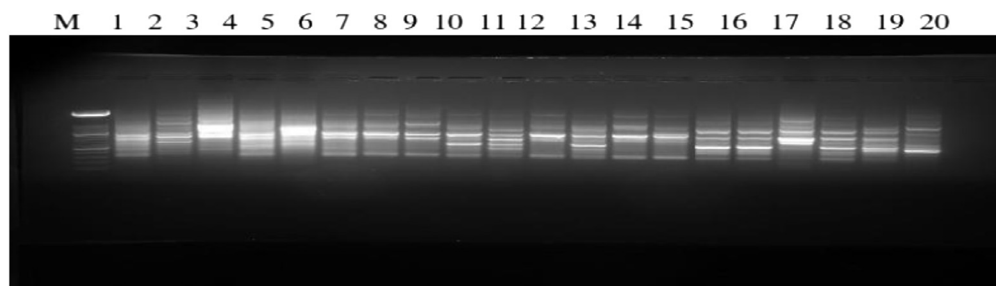


Fig. 1 The Random Amplified Polymorphic DNA (RAPD) for *S. Mutans* isolated using primer P1. *M molecular weight marker (100 base pairs DNA Ladder.Roche) Lanes 3,5,10,16,18,8,12,14,15,20 represent amplification pattern obtained from S.mutans isolated from Caries active normal children. Lanes 2,4,7,9,11,13,1 6,17,19 represent amplification pattern obtained from S. Mutans isolated from Caries active mentally retarded children.*

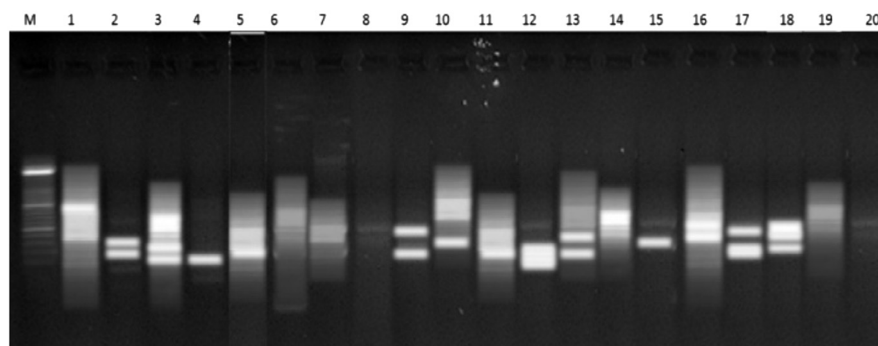


Fig. 2 The Random Amplified Polymorphic DNA (RAPD) technique was performed to study DNA of the *S. Mutans* isolated using primer P2. *M molecular weight marker (100 base pairs DNA Ladder.Roche), Lanes 3,5,10,16,18,8,12,14,15,20 represent amplification pattern obtained from S. Mutans isolated from Caries active normal children. Lanes 1,6,17,19 2,4,7,9,11,13 represent amplification pattern obtained from S. Mutans isolated from Caries active mentally retarded children.*

Table 1 *Streptococcus mutants* count in normal children and mentally retarded children.

GROUPS	N	MEAN	STANDARD DEVIATION	P VALUE	t value
NORMAL CHILDREN	20	111.6500	21.04700	0.278	1.103
MENTALLY RETARDED CHILDREN	20	102.6500	29.80864		1.103

4. Gel images

See Figs. 1–3.

5. Discussion

The relation between dental caries and *S. Mutans* is well established. The human oral cavity is a diverse environment with a combination of hard and soft tissues encompassing a total area of 215 cm^2 bathed in saliva (Auschill et al., 2001).

When a new born has 24 h of life, the so called "pioneer bacteria", frequent gram positive colonizers of oral cavity i.e. *Streptococcus* and *staphylococcus* are established (Cephas et al., 2011). Among all these two *streptococci* are of special interest in cariogenesis. Over the past few decades, extensive research has provided significant information regarding the connection between dental caries and salivary bacteria, Hence in our study we have used saliva for *Smutans* collection (Guo and Shi, 2013).

There are four serotypes in *S. Mutans* i.e., c, e, f, and k. A study was conducted by Mary et al. on molecular identification and genotyping of *Streptococcus Mutans* from saliva samples of children in Medellin, Columbia and it was concluded that the prevalence of *S. Mutans* was lower than the previous studies done with similar demographic characteristics and also *Streptococcus Mutans* serotype c, f and k were found in children with caries but not in the group without caries (Arévalo-Ruano et al., 2014).

Over the past two decades, many studies have been conducted to determine the genotypic diversity of *S. mutans* but, the relationship between its molecular diversity and the specific species that causes dental caries is yet to be understood. The world of dentistry revolves around dental caries and therefore its implication in the improvement of therapeutic approaches to treat special children is the need of the hour. Children with special health care needs are those who need extra care due to disability in the form of physical, medical or intellectual alterations. Several investigations have tried to correlate *S. Mutans* colonization levels with dental caries incidence, but

Table 2 Distribution of RAPD amplification pattern using primer P1, P2 and P3 among caries active normal children and caries active inactive children.

Distribution of RAPD amplification pattern using primers P1 P2 P3	No of genotypes isolated from each group examine	
	Mentally retarded (N-20)	Normal children (N-20)
Primer 1	80%	85%
	16	17
Primer 2	90%	100%
	18	20
Primer 3	20%	95%
	4	19

Table 3 The total number of different genotypes found in all subjects.

	RAPD amplification using primers P1, P2 and P3		
	Primer 1	Primer 2	Primer 3
No. of individuals – 40	85% 35/40	95% 38/40	57% 23/40

there are very few studies done on institutionalized disabled persons.

It had been observed that children harbor one to five distinct genotypes of *Streptococcus Mutans* at different ages (Emanuelsson et al., 1998). Information regarding the stability of microbial colonies in the oral cavity could explain the initiation of dental caries. Klien et al. identified a total of 52 distinct genotypes for normal children, but children with intellectual disability may have a less number of them. However, a tendency towards the effective stability of genotypes may differ from child to child (Klein et al., 2004). Oral examination in this study showed high DMFT index in ID children than the normal ones. Matee et al. not only found a significant relationship between *S. Mutans* levels and dental caries index, but also observed high levels of this microorganisms in children who had no carious lesions, which suggests that, the presence of cariogenic bacteria does not necessarily mean high caries activity as this is a multifactorial pathology (Matee et al., 1992).

A primer is a short single strand of RNA or DNA (generally about 18–22 bases) that serves as a starting point for DNA synthesis. It is required for DNA replication (Alberts et al., 2002). The RAPD technique is based on the polymerase chain reaction (PCR). A target DNA sequence is exponentially amplified with the help of arbitrary primers (Welsh and McClelland, 1990).

Deoxyribonucleic acid (DNA) extraction is the process by which DNA is separated from proteins, membranes, and other cellular material contained in the cell from which it is recovered. This extraction can be one of the most labor-intensive parts of DNA analysis (Elkins, 2012). There are 2 methods.

1. Biochemical and
2. Molecular i.e. based on nucleic acids

All the biochemical methods can hardly guarantee exquisite sensitivity due to features like bacterial interference or the presence of metabolically and physiologically altered bacterial cells such as the small colony variant (Botstein et al., 1980).

DNA-based diagnostics can circumvent these problems simply, due to the fact that DNA isolation from samples is experimentally simple (VenBelkum et al., 2000). Currently, many other methods like allele specific oligonucleotide hybridization, allele specific amplification are used to detect the genetic variations in human beings but RAPD (Random amplified polymorphic DNA) is preferred in the present study due to the requirement of minute amount of genomic DNA to carry out PCR technique and it essentially scans a genome for these small inverted repeats and amplifies intervening DNA segments of variable length (Redmo Emanuelsson et al., 2003).

Our results showed that this is the most convenient way of identifying *S. Mutans* isolates from the culture. Truong, et al. (2000) and Gronroos and Alaluusua (2000) suggested the same

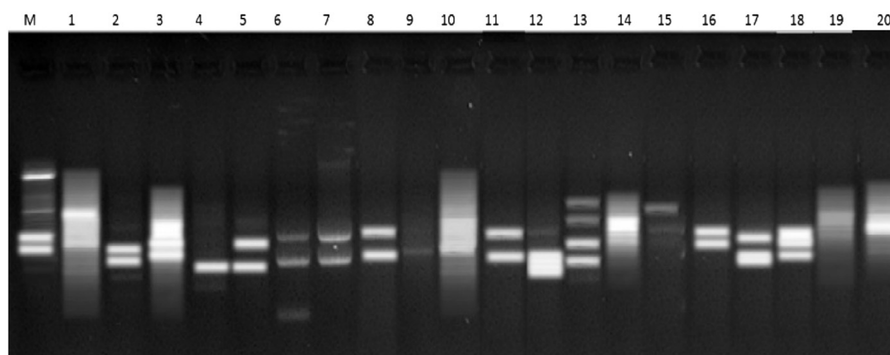


Fig. 3 The Random Amplified Polymorphic DNA (RAPD) technique was performed to study DNA of the *S. Mutans* isolated using primer P3. M molecular weight marker (100 base pairs DNA Ladder.Roche), Lanes 3,5,10,16,18,2,4,7,9,11,13,8,12,14,15,20 represent amplification pattern obtained from *S. Mutans* isolated from Caries active normal children. Lanes 1,6,17,19 represent amplification pattern obtained from *S. Mutans* isolated from Caries active mentally retarded children.

conclusion and added that the technique is a suitable and reliable method for epidemiological studies on *S. Mutans*. In the present study the DMFT score was high in intellectually disabled children when compared to that of normal children, this is in accordance with the study done by Al-Qahtani and Wyne (2004) in which there was three times increase in mean dmft in 6–7 year old female intellectually disabled children when compared to that of normal children and four times increase in mean DMFT in female ID children when compared to that of normal children. We believe that this study clearly demonstrated the complexity of Mutans streptococci colonization in the same cavity where genotype encoding primer 2 was present in mostly in all the children's oral cavity 95% of children (Table 2). Although a genotype of *S. Mutans* RAPD pattern encoded by Primer P2 was present in almost all the individuals' amplification pattern encoded by Primer P1 was present in only 85% of children in both the groups (Table 2). This finding was augmented by Redmo Emanuelsson et al. (2003) as they reported the possibility of several different genotypes of *S. Mutans* to colonize the tooth sites in the same oral cavity (Redmo Emanuelsson et al., 2003).

Whereas genotype encoding Primer P3 was present in only 20% (Table 2) of ID group this might be because of the secretory IgA and over expression of the Super oxide dismutase (SOD) which is present in all aerobic organisms and some anaerobic organisms, it catalyses the conversion of superoxide radical to oxygen and hydrogen peroxide and plays an important role in cellular defense against oxidative stress (Chandrashekhar and Bommangoudar, 2018; Lee et al., 2004).

This type of actions are present in Autistic and down's syndrome children with mild disability who were the subjects in the Mentally retarded group of our study.

Although cognitively disabled children have complex flora, it is possible to overlook the transient genotypes in a single pooled sample. Hence further studies are needed to correlate their salivary chemistry with the bacterial genotypes. Studies have already shown that due to the magnitude of systemic medical conditions oral health is most often neglected in special children. Therefore, in an attempt to find better solutions to improve the oral health of such children this study could prove to be instrumental in its own way for future research.

6. Conclusion

In accordance with the results of our study, there was a significance difference in Genotypic diversity of *S. Mutans* between caries active normal children and caries active Mentally retarded children.

7. Recommendations

Further research is needed to compare the salivary chemistry and its association with a genotypic diversity of *S. Mutans*.

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