Early and accurate detection of bacterial isolates from dental plaque in subjects with primary, mixed, and permanent dentition by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry technique

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Abstract:
Background: Bacterial colonization of dentition in different age groups can impact prognosis in different dental diseases. Latest diagnostic technique such as matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) is increasingly being used for accurate identification of bacteria. This study was undertaken to evaluate the MALDI-TOF MS technique to identify bacterial pathogens from dental plaques in subjects with primary, mixed, and permanent dentition. Materials and Methods: The study included 150 subjects of different age groups and were divided into three groups - Group A: Subjects with primary dentition (n = 50), Group B: Subjects with mixed dentition (n = 50), and Group C: Subjects with permanent dentition (n = 50). Subgingival dental plaque samples were collected from buccal and lingual surfaces of premolar and molar teeth. Clinical parameters such as gingival index were recorded. Samples were cultured in routine aerobic and anaerobic medium. Bacterial growths were assessed by semiquantitative methods. Bacterial isolates were confirmed by MALDI-TOF MS technique. Results: MALDI-TOF MS detected all the culture-grown bacteria. In primary dentition group, purple and yellow complex bacteria predominated. Streptococcus spp. was the predominant bacteria (51%) followed by Escherichia coli (19%) and Veillonella spp. (19%). In mixed dentition and permanent group also, Streptococcus spp. was predominant (46%) followed by Veillonella spp. (24%) and E. coli (19%). However, in both groups, orange complex bacteria (bridge complex) such as Prevotella nigrescens and red complex bacteria (Porphyromonas gingivalis, 3%) were seen. For majority of bacteria, the load increased with age. Conclusions: The bacterial isolates showed a distinct age-specific colonization. The use of advanced technique such as MALDI-TOF MS is helpful in the detection of periodontal pathogens, and the effective oral health programs can be implemented to minimize the risk of periodontal diseases.

Key words:
Bacteria, dentition, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, periodontal disease

INTRODUCTION

Each anatomic site in human body has a unique niche for certain bacteria. After birth, the niches form when complex person-to-person and person–environmental interaction occurs.[1] Early close household contacts may be the source of acquisition of bacterial colonization for infants and children.[2] Understanding of age-related initial colonization of periodontal pathogens, especially Porphyromonas gingivalis and Tannerella forsythia, would provide a potentially useful diagnostic tool in the early detection and prevention of periodontitis in healthy individuals. The importance of this in the context of age-related.....
initial colonization of periodontal pathogens is derived from the premise that some earlier oral colonizers may facilitate the biofilm formation of Treponema denticola. 

One study has reported that initial colonization by five selected periodontal pathogens, including Campylobacter rectus, Aggregatibacter actinomycetemcomitans, P. gingivalis, Prevotella intermedia, and T. forsythia, could be detected in distinct age-related groups from newborns to elders.\(^{10}\) Colonization of P. intermedia and T. forsythia appears to start at earlier ages, during the period when permanent teeth start erupting (6–12 years old), whereas colonization by P. gingivalis appears to colonize when mixed dentition shifts completely to permanent dentition (>13 years of age).\(^{11}\) A. actinomycetemcomitans is known to be an early colonizer and strongly associated with localized aggressive periodontitis in children. A study has found A. actinomycetemcomitans in 20% of periodontitis group as compared to 4.8% in healthy group in children of 2–12 years of age (primary and mixed dentition).\(^{12}\)

Socransky et al. classified these pathogenic bacteria according to their association with severe forms of periodontal disease.\(^{3,4}\) P. gingivalis, T. forsythia, and T. denticola are all categorized in the red complex. T. denticola, a motile and highly proteolytic Gram-negative oral spirochete, is thought to be a late colonizer during plaque biofilm formation. Bacteria of the orange complex, such as C. rectus, P. intermedia, Eikenella corrodens, and Parvimonas micra, are more tightly associated with red complex, and they are associated with the initial colonization of red complex bacteria in the oral cavity. Bacteria of other complexes (purple and yellow) are also usually early colonizers of oral cavity. A study of children aged 3–12 years showed early colonization in the primary dentition by yellow complex bacteria (Streptococcus spp.) and purple complex bacteria (Veillonella spp.) whereas red complex bacteria (P. gingivalis, T. forsythia, and T. denticola) were found in mixed dentition subjects.\(^{8}\)

Identification of age-related bacterial colonizers can help in detection of potential pathogens and early intervention to prevent disease. Different microbiological techniques are available for identification of the bacteria. The traditionally conventional culture and identification of bacteria are usually done in laboratories, but these methods are cumbersome. However, bacterial load by colony counting is an important parameter in the prediction of disease progression.

The introduction of advanced diagnostic technique such as the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has improved the diagnostic ability of laboratories. This method uses laser vaporization technique and detects charge/mass ratio of the cell wall proteins. It compares the results and matches with large database. This method helps to rapidly identify the causative pathogens from culture. The present study was undertaken to evaluate MALDI-TOF MS to identify bacterial pathogens from dental plaques in subjects of primary, mixed, and permanent dentition to determine the age-related bacterial population.

**MATERIALS AND METHODS**

The study was done in preprimary, primary, and high-school children over 1-month period. The study included 150 subjects. Informed consent was taken from the parents and school authorities. Ethical clearance was taken from institute ethical committee. The following subjects were excluded from the study: (1) subjects with uncontrolled systemic diseases; (2) subjects with any immunodeficiency conditions; (3) subjects who had taken antibiotics within the 6 months before the clinical and microbial examination, (4) subjects who use orthodontic devices; (5) subjects who underwent periodontal treatment 12 months before the beginning of the study.

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**Microbiological study**

The samples were placed into sterile tubes filled with 1.95-ml prereduced culture medium (Robertson’s Cooked Meat medium) such that only minimal air volume remained. Within ½ h, the samples were sent for microbiological study.

The samples were inoculated in serial dilutions onto sheep blood and Wilkins-Chalgren agar. Blood agar plates were incubated in air enriched with 10% CO\(_2\) whereas Wilkins-Chalgren agar plates were placed in anaerobic jars for up to 7 days at 37°C. Anaerobic conditions were created by Anaeromate System. Representative colonies were isolated according to growth morphology, pigmentation, and hemolysis. All isolates were subjected to various microbiological methods for identification. The isolates of bacteria were identified by Gram staining and biochemical tests. Their number was counted in colony-forming unit (CFU) by serial dilution method as described in a previous study.\(^{7}\)

The bacterial isolates were subjected to MALDI-TOF MS assay. This assay was performed in VITEK-MS (BioMerieux) system. Pure cultures of bacterial isolates were processed. First, the provided disposable plate containing 48 spots were taken. With a wooden applicator, bacterial spots were prepared on the plate. The spot of control bacterial strain (Escherichia coli ATCC 8739) was prepared on the control spot. Then, the spots of test isolates were made. After that, 1 µl of provided matrix (α-cyano hydroxy 4-cinnamic acid) was put on each spot by a micropipette.\(^{9}\)

After all the spots of bacterial isolates were made, the plate barcode was scanned by prep-station scanner. The details of the isolates were entered in the prep-station computer. Then,
the details were sent to acquisition-station computer. Again, the barcode of the plate was scanned. The plate was put in the VITEK-MS and machine was started. The generated mass spectrums were matched with the database and the bacterial isolate was identified.

**Statistical analysis**

Categorical groups were summarized in number (n) and percentages (%). They were compared by Chi-square test. Groups were compared by one-way analysis of variance (ANOVA) test, and significance mean difference between the groups was done by Tukey’s honestly significance difference (HSD) post hoc test. P < 0.05 was taken statistically significant. Analysis was performed on SPSS Statistics Windows (Version 17.0., SPSS Inc., IBM, Chicago, IL, USA).

**RESULTS**

Table 1 shows the demographic data of the subjects under study. The male and female percentage was 58% versus 42%; 64% versus 36%; and 66% versus 34% in primary, mixed, and permanent group, respectively.

Majority of children were from low socioeconomic status.

MALDI-TOF MS detected all the culture-grown bacteria. As shown in Table 2 and Figure 1, in primary dentition group, *Streptococcus* spp. (*Streptococcus oralis* and *Streptococcus mitis*) were the predominant bacteria (51%) followed by *E. coli* (19%) and *Veillonella* spp. (19%). In mixed dentition group also, *Streptococcus* spp. were predominant (46%) followed by *Veillonella* spp. (24%) and *E. coli* (19%). *P. gingivalis* (3%) and *Prevotella nigrescens* (3%) were also detected. In permanent dentition group, *Veillonella* spp. was predominant (31%) followed by *Streptococcus* spp. (23%), *E. coli* (23%), and *P. micra* (15%). In this group also, *P. gingivalis* (3%) and *P. nigrescens* (3%) were detected. In all groups, no *A. actinomycetemcomitans* was detected.

The bacterial load was determined by semiquantitative culture method. The mean bacterial load in different groups is shown in Table 3. The mean bacterial load of *Streptococcus* spp., *Veillonella* spp., *P. nigrescens*, and *P. gingivalis* increased gradually in the three groups. However, the mean bacterial load of *E. coli* decreased and that of *P. micra* remained same.

One-way ANOVA with post hoc Tukey’s HSD test was done to detect the significance difference level of the bacterial load between groups. The Q statistic was 0.4330, 0.8660, and 0.4330 between primary and mixed group, primary and permanent group, and mixed and permanent group, respectively (nonsignificant).

**DISCUSSION**

Colonization of different bacteria in the oral cavity and dental plaques can have a role for subsequent development of different dental diseases. Early and accurate diagnosis of different bacteria of normal and pathogenic potential is extremely important. The type of bacteria and load can accurately predict the course of different periodontal diseases. Advanced diagnostic techniques such as MALD-TOF MS can help in the achieving the purpose.

In this study, subjects of different age groups having primary, mixed, and permanent dentition were studied. Both sexes were
included in the study, but the number of males was higher than females in all age group. However, we could not find any gender-specific bacteria in our study.

Oral streptococci (yellow complex) and E. coli were the predominant bacteria followed by Veillonella spp. (purple complex) in primary dentition group. Similar findings were reported by Papaioannou et al. who reported Streptococcus spp. and Veillonella spp. as predominant bacterial species in all age groups. These bacteria are commonly found in gastrointestinal tract and environment, so oral cavity comes in contact with them first. These bacteria are transient colonizers of primary dentition and rarely associated with periodontal diseases. However, S. mitis and S. Streptococcus mutans are usually associated with dental caries typically seen in young children. We did not find any red or orange complex bacteria in this age group. Similar findings were observed by Frisken et al. The orange complex bacteria P. micra was detected in 8% children. This bacterium is known to be closely associated with subsequent colonization by red complex bacteria.

The prevalence of different bacteria showed similar trend in mixed dentition group, where Streptococci spp. were predominant followed by Veillonella spp. and E. coli. However, we detected P. micra, P. nigrescens (orange complex), and P. gingivalis (red complex) in this age group (5%, 3%, and 3%, respectively). The bacteria in the green- and orange-associated complexes are able to adhere to the pellicle by means of fimbrae and thereby avoid being flushed out with the gingival crevicular fluid. The orange complex bacteria are also called as “bridge species” form a link between the early colonizers and the highly pathogenic bacteria of the red complex. Through their metabolism, these bacteria also create the living conditions for the strictly anaerobic bacteria of the red complex and their colonization of the sulcus. Some authors have reported high prevalence of P. gingivalis and even very early colonization. Other studies have reported low prevalence of P. gingivalis (8.3%) but high prevalence of P. nigrescens (48.3%) in the mixed dentition group. This indicates early colonization by orange complex bacteria in mixed dentition group which subsequently leads to colonization by red complex bacteria. In this group of children, strict oral hygiene and regular dental checkup are must to prevent subsequent periodontitis.

In the permanent dentition group, the orange and red complex bacteria (21% combined) were seen in greater amount than those of primary (8%) and mixed dentition group (11%). Higher prevalence of orange complex (70%) and similar prevalence of red complex (8%) have been reported by Carrouel et al. in permanent dentition group. However, in that study, the subjects were young adults. Aas et al. reported high prevalence of Veillonella spp. and Streptococcus spp. and low prevalence of Prevotella spp. In their study, Papaioannou et al. reported distinct age-related increase in P. gingivalis and T. forsythia.

Another prevalent bacterium in all age groups was E. coli. Although considered as nonoral bacterium, it can colonize dental plaque. Souto et al. reported high prevalence of E. coli in healthy sites in periodontitis patients. Other studies also reported E. coli in healthy sites of oral cavity. Hence, this may implicate good oral health.

In our study, the oral pathogen A. actinomycetemcomitans was not detected in any groups. However, large sample population may have been yielded this bacterium as this pathogen may be low in our study population.

Another finding was that the mean bacterial load increased for all bacteria except E. coli for which the load decreased. In their study, Papaioannou et al. reported similar increase of pathogenic bacteria, especially Streptococcus spp. and P. gingivalis in older children. It is possible that with increasing age, bacteria population increases by subsequent attachment to biofilms.

There are many diagnostic methods to identify the oral anaerobic bacteria. Conventional microbiological identification methods are cumbersome and time-taking. Polymerase chain reaction (PCR) is highly sensitive and specific method, but it is expensive and needs expertise. Majority of the quoted studies utilized the molecular methods including PCR and they identified the bacteria to species level. In this scenario, newer identification method such as MALDI-TOF MS can play a role in rapid and accurate diagnosis of dental plaque bacteria. The early and accurate detection of potential pathogens of dental plaques is essential so that strict oral hygiene can be maintained to prevent periodontitis. Conventional identification methods take longer time and all anaerobic species cannot be determined by these methods. These methods are also costly and labor intensive. MALDI-TOF MS is helpful in this situation because this method is very rapid (takes 15 min) and very accurate because it utilizes the proteomics of bacteria which are not altered. The average cost per test also very less as compared to conventional methods. For this purpose, this technique is replacing the conventional methods in bacterial identification.

Stingu et al. used MALDI-TOF MS identification in 84 isolates of anaerobic bacteria previously identified by phenotypic methods. Furthermore, they compared MALDI-TOF MS results with those of 16S RNA PCR in nine reference isolates. They found comparable results of MALDI-TOF MS with 16S rRNA PCR and superiority of MALDI-TOF MS to biochemical identification method. In this study also, MALDI-TOF MS led to early and accurate identification of microflora in dental plaques so that proper infection control measures advised to prevent pathogenic bacteria colonization. Zhang et al. conducted a study in which bacteria were isolated and cultured from caries patients and each isolate was identified by both 16S rRNA sequencing and MALDI-TOF MS. The identification results obtained by MALDI-TOF MS were concordant at the genus level with those of conventional 16S RNA-based sequencing for Lactobacilli (62/70). In our study also, MALDI-TOF MS led to early and accurate identification of microflora of dental plaques in different age group children.

The bacterial isolates showed a distinct age-specific colonization. Red complex bacteria are seen more in older age group.

CONCLUSION

New diagnostic technique such as MALDI-TOF MS can be utilized for early, cheap, and accurate identification of early colonization of pathogenic oral bacteria. It will help in proper infection control measures to prevent periodontitis and other oral diseases. Further studies are required in this area.
Acknowledgement
We like to thank all participants in the study.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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