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Analysis on Anaerobic Bacteria in Dental Plaque among Healthy Asymptomatic Subjects- Microbiological Perspective.

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ABSTRACT:
Dental diseases resulting from oral infections represent a major health concern. We aimed to investigate the scope of anaerobic organisms in the oral cavity of an apparently healthy asymptomatic housekeeping community at a tertiary care setting in Chennai India, between Februaryand November, 2019. **Sample:**One hundred and fifty-eight housekeeping staff were screened for dental plaques, among which50 individuals were investigated for anaerobic colonizers from dental biofilms. **Method:**Anaerobic culture was performed on Robertson's cooked meat media, representative samples were processed for 16S rRNA sequencing. **Result:**Pepto streptococcus sp. was most prevalent followed by Bacteriodes sp., Peptococcus variabilis, Clostridium sp. and C. perfringens. Vellionella sp.; S. moorei was confirmed through 16S rRNA sequencing,and GenBank accession numbers were generated. **Conclusion**: Adequate awareness could aid in prompt diagnosis, treatment of chronic oral diseases that would risk the development of life-threatening conditions, especially during the prevailing COVID-19 pandemic. Overuse of face masks due to the prevailing SARS-CoV-2 pandemic skews attention towards development of infections by opportunistic organisms.

Keywords: Oral infections, Anaerobic bacteria, Antibiotic resistance, Dental plaque, Opportunistic pathogen

I. INTRODUCTION:

Oral hygiene is the primary key for many infections, the need of the hour during the SARS-CoV-2 pandemic. The oral cavity offers an ecological niche for a wide array of aerobic as well as anaerobic microorganisms. Dental disease encompasses both oral infections as well as cancer representing a major global health concern. The consequences of poor oral hygiene can be witnessed at the personal population, as well as at the health system levels. The World Health Organization (WHO) recognizes oral hygiene as an integral part of the general well-being of an individual. Prolonged use of face masks during the SARS-CoV-2 pandemic has led to a surge in oral and dental conditions due to anaerobic infections by opportunistic pathogens warranting urgent public health measures. Dental caries and periodontal disease can deteriorate community health leading to decreased economic productivity, can act as a significant risk factor for other systemic health ailments [**WHO, 2003].**

Dental caries represents a key cause of development of chronic disease among children and adults worldwide. Dental Caries is caused by bacterial colonizers of the oral cavity forming plaques that could benefit bacterial survival leading to the origin of dental biofilms. The formation of dental plaque is attributed to the interaction of specific bacteria with constituents present in the diet. The knowledge of bacterial flora involved in the formation of biofilm in the population is key to prevention of oral and dental infections including periodontitis, oral cancer, dental caries Anaerobic bacteria due to their fastidious nature, laborious workload for recovery from clinical specimens are almost always overlooked in clinical laboratories. The COVID-19 pandemic has imposed the possibilities for the emergence of oral infections due to immunocompromised state and anaerobic environment generated due to the prolonged use of face masks. The prevailing pandemic situation underlines the need for awareness on dental/oral health routinely to prevent the transition of the normal flora into opportunistic pathogen may be due to excessive use of face masks.

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Biofilms or plaque is the result of surface–adherent population of microorganisms together with waste and extracellular matrix materials. Streptococcus mutans (S.mutans), the principle cariogen for dental caries, reportedly co-exists with ~500 other bacterial species as an interactive community in the dental biofilm (**Berezow et al., 2011**). Biofilm serves as a shield to protect bacteria from the host immune system, for instance antimicrobial peptides and antibiotics.The organisms in the biofilm environment confers certain properties to bacteria that does not occur in the normal state, a fact that explains the importance of recognizing dental plaque as a biofilm and not as bacteria in the planktonic state. Dental biofilms appear to be important as bacteria adhere to dental implants, tissues and other surfaces of the oral cavity. The oral cariogenic bacteria interact with the host via co-aggregation, metabolic exchange, cell–cell communication, and exchange genetic material, therefore biofilm represents a major therapeutic target for resistance development in dental ailments **(Jacob et al.,2006)**Evidence suggests that the recently identified S. moorei is key to development of halitosis (**Haraszthy et al., 2008; Hiranmayi et al., 2017).** Literature suggests that failure to direct therapy against these organisms often lead to far-reaching clinical conditions and treatment failures **(Berezow et al., 2011).** Lack of interest to screen anaerobic oral bacteria stems from laborious specimen collection procedures, transportation, and complex cultivation methods for recovering the etiological agents in diagnostic laboratories. Here, we studied the prevalence of cultivable anaerobic oralbacteria amongthe healthy, asymptomatic housekeeping staff in a tertiary care setting, and emphasized the application of 16S rRNA as a diagnostic tool to identify anaerobic bacteria.

The prevailing pandemic situation underlines the need for awareness on dental/oral health routinely to prevent the transition of the normal flora into opportunistic pathogen which may be due to prolong duration to use face masks which leads to create anaerobic environment.

II. MATERIAL AND METHODS

Subjects and Ethics Approval

The prospective study was done at the Department of Microbiology, Sri Muthukumaran Medical College Hospital and Research Institute (SMMCH&RI) Chikarayapuram near Mangadu for a period of 5 months from February to November 2019. The study was funded by ICMR SDS and ethical clearance was obtained from the Ethics Committee SMMCH&RI. The study was carried out following approval of the protocols by the Medical Ethics Committee (MEC) (Ref. No. 938.42) for ethical issues. Written consent was obtained from all the participants before study enrollment. The study was carried out in compliance with good clinical practice, including the International Conference on Harmonization Guidelines and the Declaration of Helsinki.

A total of 158 healthy housekeeping staffs were selected through an organized free dental checkup. The selection criteria were followed strictly. All the participants were given a questionnaire to analyze the demographic data on oral health. Of all the individuals screened, 50 volunteered participants with tartar biofilms were enrolled into the study. The participants were explained about the project and their demographic data comprised age, gender, socioeconomic status, education, food habits, past and present history of dental problems (if any), oral hygiene habits, and lifestyle. The questionnaires were filled by the participants and duly signed. Various physical examinations like decayed teeth, missing and filled surfaces (DMFS), dental plaque index and hygiene index were performed (Greene and Vermillion, 1964). Light and exploration with dental mirrors were used to examine the oral cavity according to the standard WHO recommendations

Samples

The tartar materials were recovered as samples from the tooth region (anterior and posterior) of incisors, canine and molars using a sterile hand scaler. The samples were inoculated into BHI broth, Robertson's cooked meat medium, and peptone water. All the samples were collected aseptically in the dental OP and transported to the microbiological laboratory for processing.

Anaerobic cultivation

The isolation of anaerobic bacteria was performed as per standard procedures: Following the inoculation of the tartar material, the RCM media was incubated at 37^{o}C for 72 hrs. Later, 10µl of RCM broth was inoculated on sheep blood agar (SBA), chocolate agar and SBA supplemented with neomycin (30mg/L). The neomycin blood agar plates were incubated at 37^{o}C for 72 hrs in McIntosh and Fildes' anaerobic jar with AnaeroGas pack (Himedia, India). On day 4, the plates were examined for the growth of colonies. The colonies were confirmed as anaerobic by examining aerotolerance. The identification of colonies was based on Gram staining microscopy, morphological appearance and biochemical characterization. The Kirby- Bauer disc diffusion method was performed

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to determine antibiotic sensitivity according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Presence of 10^5 colony forming units per liter (CFU/ml was suggestive of a diseased state.The aerotolerant samples were processed for 16S rRNA gene sequencing.

16S rRNAgene sequencing

The 16S rRNA gene sequencing was used for the identification of anaerobic isolates, according to the protocol using a commercial DNeasy Blood and Tissue Kit (Qiagen, Germany) and DNA extraction was performed from fresh bacterial isolates.

PCR reaction was performed as described by **Weisburg et al (1991)** targeting the 16S rRNA gene for the isolate with the reaction volume of 25µL with a broad range of 16sRNA primers "16S Fp-5'- AGAGTTTGATCCTGGCTCAG-3' and 16S Rp-5'ACGGCTACCTTGTTACGACTT-3' (10 pM of each primer), 10× PCR buffer ,10 mM of dNTP mix, 1 units Taq DNA, DNA template (0.1-1 µg of DNA) and PCR milli Q grade water. The PCR amplification was performed with 37 cycles of denaturation for 30s at 95°C starting with initial denaturation for 3min at 95°C, further steps following 55°C for 30 s and 72°C for 1 min, finally with extension at 72°C for 7 min with a known positive and negative controls in a MasterCycler (Eppendorf, Germany). Together with DNA markers the amplicons were resolved in 0.8% agarose with ethidium bromide (10mg/ml) by gel electrophoresis using Mupid-exU system (Takara Japan) using the BioGlow UV Transilluminator, and the gel was analyzed with the product size of 1500bp.

BLAST analysis

The PCR product of 16SrRNA was sequenced at Macrogen Inc. (Seoul, Korea) using ABI PRISM®BigDye™ Terminator and ABI 3730XL sequencer (Applied Biosystem, USA) with the forward and reverse primers mentioned previously. After sequencing, the sequence chromatogram files were examined for quality and low quality ends of 16S rRNA sequences was trimmed by using Bio-Edit version 7.0.9 (Isis Pharmaceuticals) or Codon Code Aligner version 4.0 software (Codon Code Corporation). The species identification of the bacteria was achieved by comparing the nucleotide sequence of 16S rRNA gene with the GenBank microbial genomes database using the Basic Local Alignment Search Tool (BLAST), (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS:

Patient Dermography:

Fig I: Showing the Age group analysis among the total number of participants in oral health screening camp and the study volunteer.

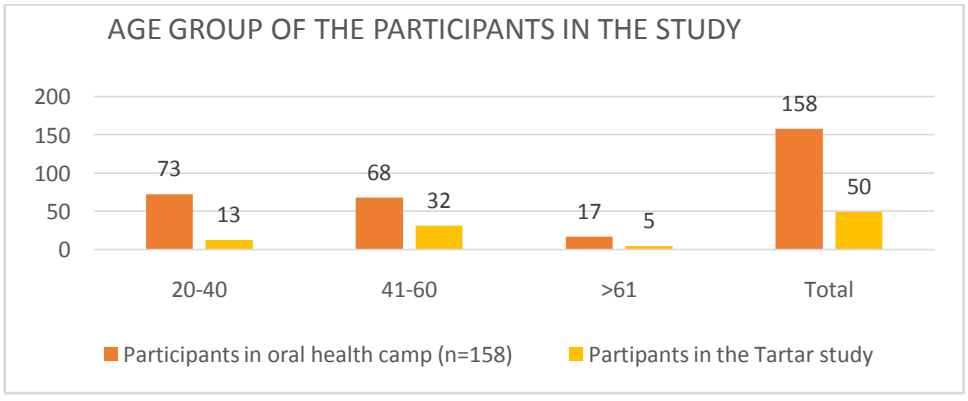

Fig:II :

The Anaerobic organisms cultivated in our study is shown the Pie Diagram

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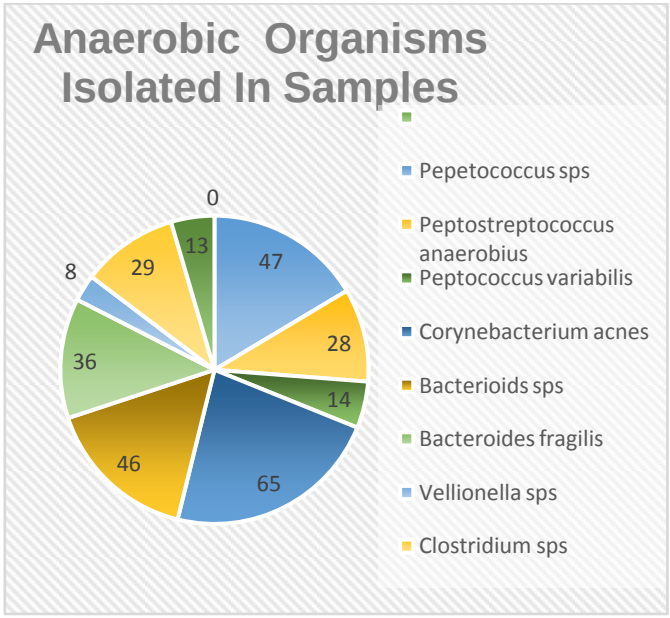
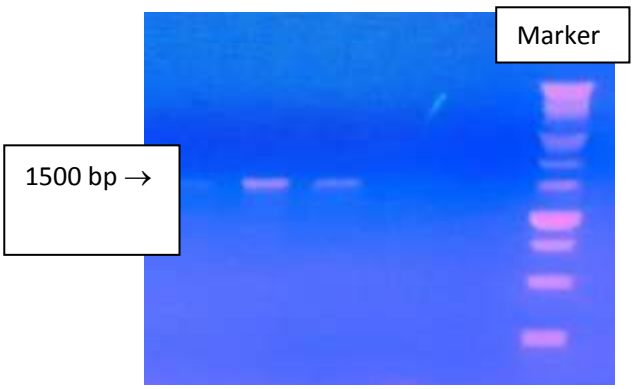


Among our anaerobic cultures, numerous isolates of Peptococcus sps, Peptostreptococcus anaerobius, Peptococcus variabilis Clostridium sps Corynebacterius acnes Bacterioid's species, Bacteroides fragilis and vellionella sps. Clostridium sps and C.perfringes were the predominant organisms. Further we were able to account isolates of Peptostreptococcus sps in 94 %, Bacteriodis sps in 92 % of samples whereas Vellionells sps could be isolated in only18% of samples.

Molecular identification of anaerobes isolated using 16S rRNA gene sequencing:

The aero tolerant samples were processed to identify and confirm the organism by 16Sr RNA gene sequencing This study with S.moorei was confirmed with GenBank accession numbers and were found with MT418895 and 1045base pairs for confirmation and identity. BLAST analysis matched the strain of Moorei strain Solobacterium moorei strain DC-3 16S ribosomal RNA gene, partial with a second sample (MT418896 and 1260 base pairs DNA linear BCT) as Solobacterium moorei` strain DC5 16S ribosomal RNA gene, partial sequence, Both the strains had 99.9% similarity. GEN BANK accession number with link is given as follows: https://www.ncbi.nlm.nih.gov/nuccore/MT418895, MT418896



99.9% sequence homology was observed in Solobacterium moorei which was identified by 16S rRNA Sequencing.

III. DISCUSSION:

The dental plaque contains >700 different microorganisms that induce rapid changes in pH, nutrient availability and oxygen tension, they are often exposed to toxic compounds in the oral cavity

(e.g. healthcare products, food additives and tobacco). Though the dental plaque-causing bacteria remain relatively stable, during this pandemic situation due to change of pH and closed environment created by wearing the face mask the altered oral homeostasis may lead to a shift in microflora as reported by **Marsh et al. (2012).**According to the National Institutes of Health (NIH) all microbial and chronic infections 65% and 80%respectively, are associated with biofilm formation.**Jamal et.al.,(2018.)**Anaerobic infections may cause serious and life-threatening infections, because of their fastidious nature since they are difficult to isolate, often overlooked. Sometimes lack of therapy to these organisms leads to clinical failure.Our study focuses the importance and urgent need to prevent oral anaerobic infections at this time, while wearing mask during the pandemic. Further, prevention of an anaerobic infections serves as a marker for prevention of complications due to bacteremia. Study identifies various oral organisms in all the healthy persons showing a variety of Streptococcal specieseg.S.mutant, S.sanguinis.,S. pneumonia, S.gordonii. Further we could see that almost all had coagulase negative Staphylococcal species with staphylococcus aureus and Peptococcus sps, Peptostreptococcus anaerobius, Peptococcus variabilis vellionella sps. C.perfringes, Clostridium sps Corynebacterius acnes Bacteroides fragilis. S. moorei. Bacterioid's species was found to be the predominant organism, our study was supported by similar Staphylococci bacterial species in dental biofilms **Alsaimary et al., (2012)** ;**Jagtap and Karkera et al., (1999)** ; **A.Devi et al.,(2012). McCombs GB et.al., (2010)** and **Kolenbrander et.al (2000)**in their studies showed a variety of bacteria including both aerobes and anaerobes. Our study supports that predominant and bulk microorganism among the anaerobes in the dental biofilms to be S. mutants which forms the initial colonizers of the tooth surface in the early biofilm community. The opportunistic pathogens plays a key role in dental infections and S. moorei has been isolated from various types of dental infections **(Downes, J., et al. 2001, RolphH. J., et al. 2001, SchirrmeisterJ.F.,et al. 2009; Zheng, G., et al. 2010).** Most common source of infections to be tooth abscess as reported by **Rune Micha Pedersen 2011; Detry Get al. 2006**). S. moorei is a strict anaerobic gram positive bacillus ,non sporing only species of the genus Solobactetium belonging to Clostridium cluster XVI with close association, due to the difficulty in identification it was placed among Eubacterium strains, now sequencing technology facilitated the identification of S. Moorei from clinical samples(**Zheng, G., et al. 2010).**Though S. moorei was once thought to be associated with halitosis (**Haraszthy et.al., 2008&Haraszthy2007;Kazor et.al., 2003**)periradicular lesions(**Schirrmeister, J. F., et al. 2009.**) and subgingival plaques(**Colombo, A. P., et al. 2009**) recent studies shows S.moorei to be associated with bacteremia from various sources like abscess from abdomen (**Lau, S. K., et al. 2006**), groin(Martin, C. A et.al.,2007). Our study emphasis the need for a closer look at S. moorei which was once considered as normal flora has been reported in infection associated with predisposing conditions like diabetes , immunodeficiency, malignancy , trauma, previous surgery where there is an opportunity for anaerobe. S.moorei to penetrates the deeper tissues. when the conditions are favourable.

S. Moorei may cause opportunistic infections and act as a pathogen which may be supported by the three major virulence factors as in other anaerobes ie, the ability to adhere to or invade the epithelial surfaces, the production of toxins or enzymes that play a pathogenic role, where this organisms, can be taken care of in the earlier stages during the pandemic period**.(Rune et al., 2011)**

Maintenance of Dental health in India is very limited due to various reasons , access, cost health care services, approaches to dental clinic is done only when the person has moderate to severe pain due to infection or discomfort. The public awareness on how the normal flora can be transformed to opportunistic pathogen due to unhygienic oral and dental practices has to be emphasized. Our study supports the Research on public dental health, will raise effective preventive measures among the individuals personal dental hygiene during the present pandemic mouth mask period, where more of anaerobic environment created may lead to oro-dental anaerobic infections.

IV. CONCLUSION:

The physical examination of biofilm formation indicates a persons unawareness on the dental hygiene. Biofilm is the place where all bacteria, fungi, or the parasite localizes and acts as an indication of the forthcoming oral infections/ diseases. The awareness campaign on dental hygine and symptoms of oral diseases could aid the individual in implementing oral hygiene, thus prevention of the disease dissemination, thereby decreasing morbidityduring the pandemic. Our studybe a support to awake the general community from dangerous pathogens leading to dental loss or

progression of further infections including prevention of cancer.

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