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Isolation, Identification and Characterisation of Dental Biofilm Forming Bacteria from Clinical Isolates and Understanding Their Potential Role in Dental Plaque Formation

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KEYWORDS

dental plaque, biofilm, oral health, acidogenic bacteria, enamel

ABSTRACT:

Dental plaque is a biofilm of microorganisms that grows on surfaces within the mouth. These bacteria are responsible for the degradation of the enamel of the teeth, leading to weaker teeth, dental health and further complications. Further health complications can occur as a possibility of the infection spreading further into the body is likely due to the high microbial load. The condition progresses gradually due to two main factors- the microbial load of the acidogenic organisms and the pH of the oral environment altered by these organisms. It is usually preventable if discovered early. In this avenue the current research attempts to identify oral pathogens responsible for causing dental plaque and potential role of their biofilms in disrupting oral and dental health. For the study, samples from the surface of tooth were collected from healthy and infected individuals. The samples were first cultured in nutrient broth followed with nutrient agar medium by serially diluting the sample broth. A subsequent subculturing generated the pure colonies of microorganisms. They were first subjected for biochemicals tests followed by culturing on differential medium. The species identification was done using 16sr RNA sequencing and the role of each organism on oral/ dental health was studied systematically. The study concluded that as dental plaques are a chronic condition and are to be considered an infection that will affect the individual's oral health and quality of life, it is important to take precautions, maintain good food and hygiene habits to decrease the possibility of dental plaque occurring.

Introduction:

Dental caries is a multifactorial disease characterized by the dissolution of dentine (hard tissues of mouth). The interaction of fermentable dietary carbohydrates, mainly sugars, with bacteria The development of dental plaque, the initial stage of dental caries caries. This disease is caused by the interplay of three factors- host factor that includes teeth composition (calcium) and saliva, microbial composition and substrates consumed through external environment (Fig 1).

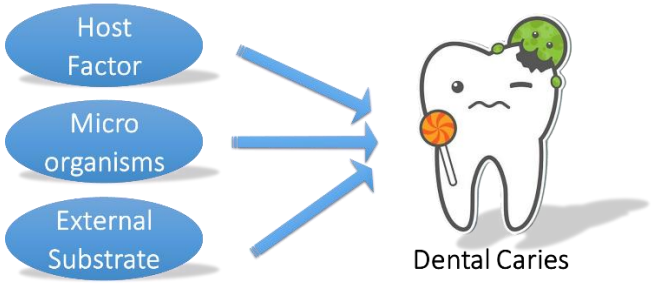

Figure 1: Factors responsible for the development of dental caries

The development of dental plaque is the initial stage of dental caries. Dental plagues are the homogeneous clumps of bacteria causing periodontal disease when they accumulate to a higher extent than the capacity of host defence (Fung and Miller 1973). As this infection

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proceeds, the pH of the dental plaque biofilm drops locally resulting in the bacteria's metabolism of carbohydrates to create increased acidic environment. When the pH falls below the critical threshold, which, for enamel is approximately 5.5, the tooth enamel demineralizes. If the equilibrium between demineralization and remineralization of the enamel is disturbed, dental enamel demineralizes when the pH falls below the critical threshold. Cavitation of the tooth structure may form over time, leading to caries lesions, if the balance between demineralization and remineralization tilts in favor of demineralization.

Dental plaque formation is the result of a complex and dynamic interaction between a number of factors. First, the host factor refers to the tooth structure's vulnerability, which includes the enamel's quality and the existence of pits, fissures, or other developmental abnormalities that could make it more likely to demineralize (Fejerskov et al. 2015). Second, microorganisms are important; the acids that propel the demineralization process are produced by acidogenic and aciduric bacteria (Marsh et al. 2009). Third, the substrate aspect has to do with eating habits, particularly consuming fermentable carbohydrates often, which give these bacteria an easy-to-access source of fuel for acid production (Selwitz et al. 2007). Finally, time is crucial because cumulative mineral loss from the tooth structure is made possible by repeated and extended exposure to acidic environments (Eaton et al. 2023). This multifactorial process can be considered a balance between protective factors, such as saliva's buffering effect, exposure to fluoride, and proper dental hygiene practices, and pathogenic aspects, such as bacteria's generation of acid.

The development of dental plaque is closely linked to the presence and activity of specific bacteria within the oral biofilm. The microbial species forming biofilms will be highly acidogenic and aciduric, meaning they not only produce acids from fermentable carbohydrates but can also survive in the low-pH environment that they create. Additionally, some species can also synthesize extracellular polysaccharides from sucrose, aiding in their adhesion to tooth surfaces and contributing to biofilm formation (Fejerskov et al. 2015; Loesche 1993).

As the carious lesion progresses into the dentin, other species become more prominent. These bacterial species like *Lactobacillus acidophilus, L. casei,* and *L. fermentum,* thrive in anaerobic conditions and contribute to the deepening and expansion of carious lesions by continuing acid production in deeper layers of the tooth (Gibbons and van Houte 1973; Selwitz et al. 2007). Their presence is a hallmark of established or advanced caries rather than early lesions.

Emerging evidence also highlights the role of Bifidobacterium species, particularly *B. dentium*, in early childhood plaque. These organisms are both acidogenic and aciduric and are increasingly recognized in studies of plaque-active children (Mantzourani et al. 2009). These findings support the ecological plaque hypothesis, which proposes that plaque results from an imbalance in the microbial community due to environmental changes, particularly a drop in pH caused by frequent sugar consumption. Under acidic conditions, acid-tolerant organisms species flourish, leading to demineralization of the tooth structure (Marsh and Martin 1992). Some bacteria, such as *S. sanguinis*, may actually provide a protective function by competing with cariogenic bacteria for attachment sites also (Beighton et al. 1991). The ecological plaque hypothesis, which postulates that caries arises from an imbalance in the microbial population brought on by environmental changes specifically, a reduction in pH brought on by frequent sugar consumption is supported by these data. As the population of microorganisms and the pH environment that the create are considered as the leading cause for the formation of dental plaque, the present study attempts to understand the threshold that differentiates conditions leading to the formation of dental plaques in comparison with healthy oral conditions. The study also tries to identify the microbial pathogen found in a local community that leading to the formation of dental plaque.

Materials and methods:

Sample collection:

The dental plaque biofilm samples were collected under aseptic conditions. Gloves and a face mask were worn during the procedure to maintain sterility. The lip or cheek of the subject was gently retracted to expose the buccal or lingual surfaces of the molars and premolars. Dental examinations were performed under natural light, using a plane dental mirror and explorer. A sterile cotton swab was used to gently scrape the tooth surface near the gingival margin, as well as interproximal areas and pits

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and fissures—common sites for plaque accumulation. The swab was then firmly rolled over the tooth surface to collect visible biofilm. The biofilm from infected individual was collected from the visiting patients using the facilities available at Sri Venkateshware dental clinic, Tarikere taluk, Chikkamagalur. The collected samples were placed in sterile phosphate buffer (pH 7.0) and processed within 2-3hr of collection along with the samples collected from healthy individual serving as control. Immediately after collection, the swab tip was placed into a sterile microcentrifuge tube containing phosphate-buffered saline (PBS) for transport to the laboratory. The samples were labelled accordingly.

Culturing of normal and infected samples:

About 60ml of nutrient agar and 4 petri plates were sterilized and the agar medium cool was cooled until it is about 45 degrees. The agar media was poured into the petri plates and was allowed to solidify. With the help of a sterile dropper about 1ml of the normal sample was added and spread with a sterile L rod to two of the plates. The same procedure was repeated with infected samples. The petri plates were labelled accordingly and incubated at 37 ºC for 24-48 hours. The different grown colonies were observed and colony characteristics were noted. The different colonies were isolated and streaked onto new agar plates with the help of a sterile inoculation loop. The petri plates were labelled accordingly and incubated at 37 °C for 24-48 hours.

Biochemical Identification of clinical isolates:

Qualitative and morphological identification studies were conducted to identify the physical and biochemical characteristics of isolated microorganisms that included Gram's staining, catalase test, methyl red vogues-proskauer test, citrate test, starch hydrolysis test, nitrate reduction test(Gerhardt 1994). Based on the results the selected isolates were cultured over differential media and antibiotic sensitivity test was conducted to identify the class of microorganisms isolated. The differential media that were selected to identify the desired organism are the MRS media (de Man, Rogosa, and Sharpe medium) and MS media (Mutans Sanguis medium). MRS Media is a differential culture medium primarily used for the isolation, cultivation, and enumeration of lactic acid bacteria (LAB), particularly Lactobacillus species. It supports the growth of fastidious organisms that require complex nutrients. MS agar is a differential medium used to isolate and differentiate oral Streptococci, especially *S. mutans*, from clinical samples like saliva or dental plaque.

Antibiotic Sensitivity Test- Abst (Bacitracin):

The test organisms that grew on the differential media were reinoculated onto fresh agar plates (differential media is used). Bacitracin disks were prepared by soaking filter paper disks in the antibiotic solution (standard concentration is 0.04 units) until the paper is completely saturated with the solution. These disks were place onto the freshly inoculated agar plates and was incubated at 37°C for 24–48 hours. The agar plates were then observed for zone of inhibition.

Characterization of isolated clinical isolates using 16Sr RNA sequencing method:

The characterization process follows following steps:

1. Extraction of DNA
2. 16s rRNA isolation and analysis
3. Bioinformatics data analysis

Extraction of DNA

To extract the DNA, I mL of each isolated strain was used, and the pellet was collected by centrifugation. After gently pipetting 20µL of Lysozyme and 200µL of TES buffer (50 mM EDTA, 100 mM Tris pH8, and 10% SDS) into the pellet, the mixture was incubated at 37 °C for 60 minutes. 20µL of proteinase K solution was then added to the mixture and left for 60 minutes. It was mixed with 250 µL of 4M sodium acetate and spun down for 5 minutes at 10,000 rpm. The clear supernatant was gathered and sent to a fresh tube. It was then placed in an ice bath and 250 µL of chloroform/isoamyl alcohol was added. The DNA pellet was obtained by re- centrifuging the material for five minutes at 10,000 rpm. They used 50 µL of TE buffer (10mM Tris pH8, 1mM EDTA) to suspend the obtained DNA sample.

16s rRNA isolation and analysis

The extracted DNA underwent 16S rDNA16S gene sequencing and analysis after PCR amplification. The 3037X DNA sequence analyser from Applied Biosystems was used to purify and sequence the amplified fragments of the 16s rRNA bacterial gene. The sequence was performed using a set of universal primers, 16SrRNA-F-27F-AGAGTTTGATCCTGGCTCAG as

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the forward primer and 16srRNA-R-1492R-CGGTTACCTTGTTACGACTT as the reverse primer. After editing the sequences using Sequence Scanner software 2. V.2.0, BLAST searches were performed to compare them with the NCBI database.

Bioinformatics data analysis

NCBI's Basic Local Alignment Search Tool (BLAST) was used to analyse all three of the acquired sequences (http://blast.ncbi.nlm.nih.gov). More dependable results were obtained by setting the search parameters to 16S ribosomal RNA (Bacteria and Archaea), filtering out uncultured/environmental samples and models (XM/XP). BLAST analysis was performed on both sequences using the forward and backward primers of each isolate, and the BLAST hit with the lowest expected value (e-value) was selected.

Results and discussion:

Collection and culturing of microorganisms from normal and infected samples:

In the present study, a diverse population consisting of 100 individuals belonging to different gender, age and food habits were considered. Depending upon colony morphology on selective and differential media. On incubating the control and infected samples of saliva samples on nutrient agar at 37°C for 24-48 hours, various bacterial cultures were observed. Most of the observed colonies were small, round and white colonies.

Biochemical Identification of clinical isolates:

Staining of the cultures isolated from caries specimens was performed to study the morphological characteristics. Gram's stained slides of all the isolates showed the presence of violet-colored cocci and bacilli species. Cocci are typically arranged in chains and all the isolates were found to be Gram positive. The results of the biochemical test performed are detailed in the below table (Table 1):

Species	Catalase test	Methyl Red Test	Voges-Proskauer Test	Citrate test	Nitrate reduction test	Starch hydrolysis	Gram's staining
CI-I	+	+	-	-	-	-	+
CI-II	-	+	-	-	+	-	+
CI-III	-	+	-	-	-	-	-
CI-IV	+	+	-	-	-	+	+

Table 1: Biochemical analysis of clinical isolates (CI)

Colony characteristics of isolates based on differential culture:

The differential media that allowed the growth of the required organisms were inoculated with the bacterial isolates to observe for growth of colonies.

MRS Media

MRS (de Man, Rogosa, and Sharpe) agar was the differential media used to screen for the presence of *Lactobacillus fermentum.* Isolate CI2 alone was observed to show growth of bacterial colonies on MRS agar plates.

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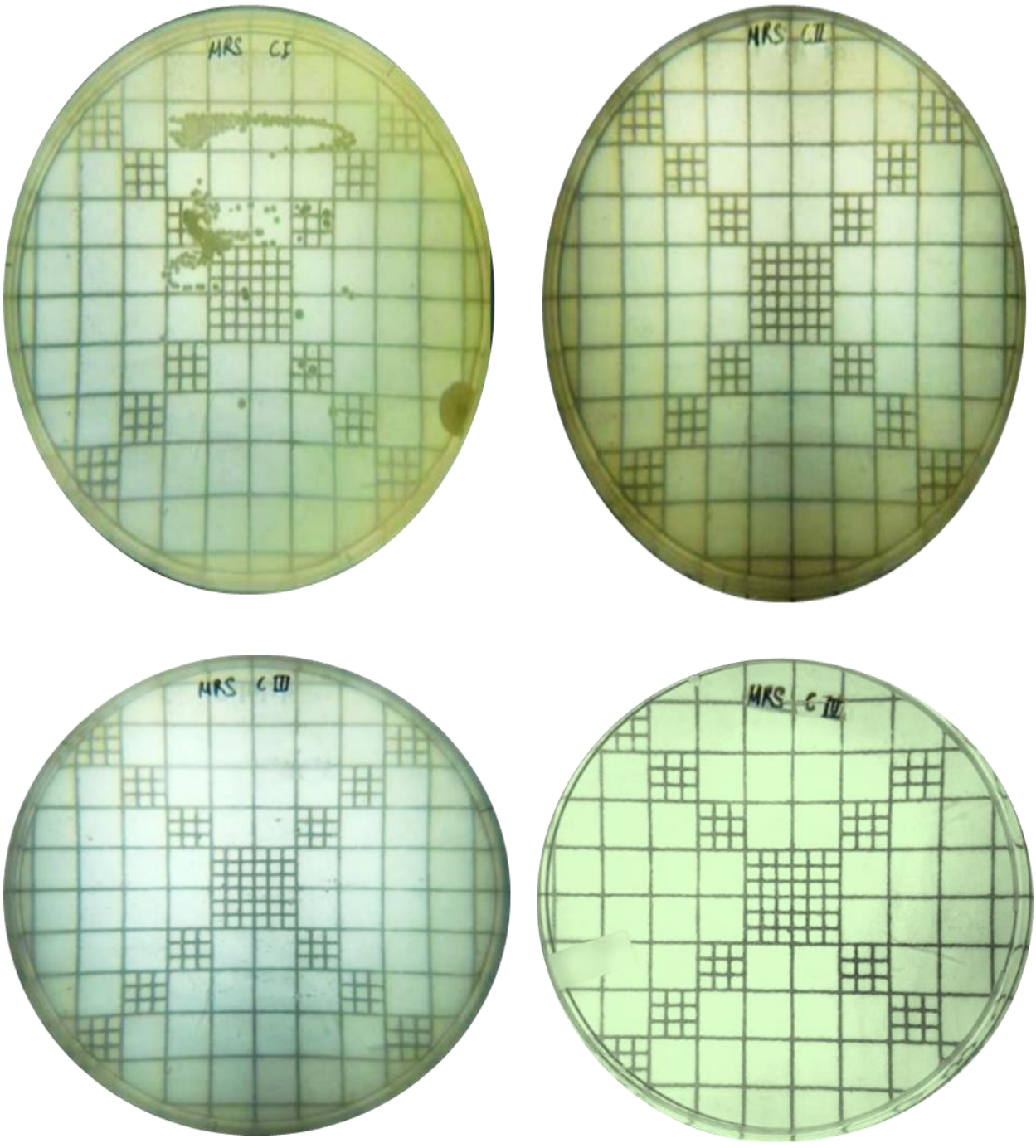

Figure 1: Isolates C1, CI2, CI3 and CI4 inoculated on MRS agar plates respectively.

MS Media

MS (Mutans Sanguis) agar was the differential media used to screen for the presence of *Streptococcus mutans.* The isolate CI1 alone was observed to show growth of bacterial colonies on the MS agar plates.

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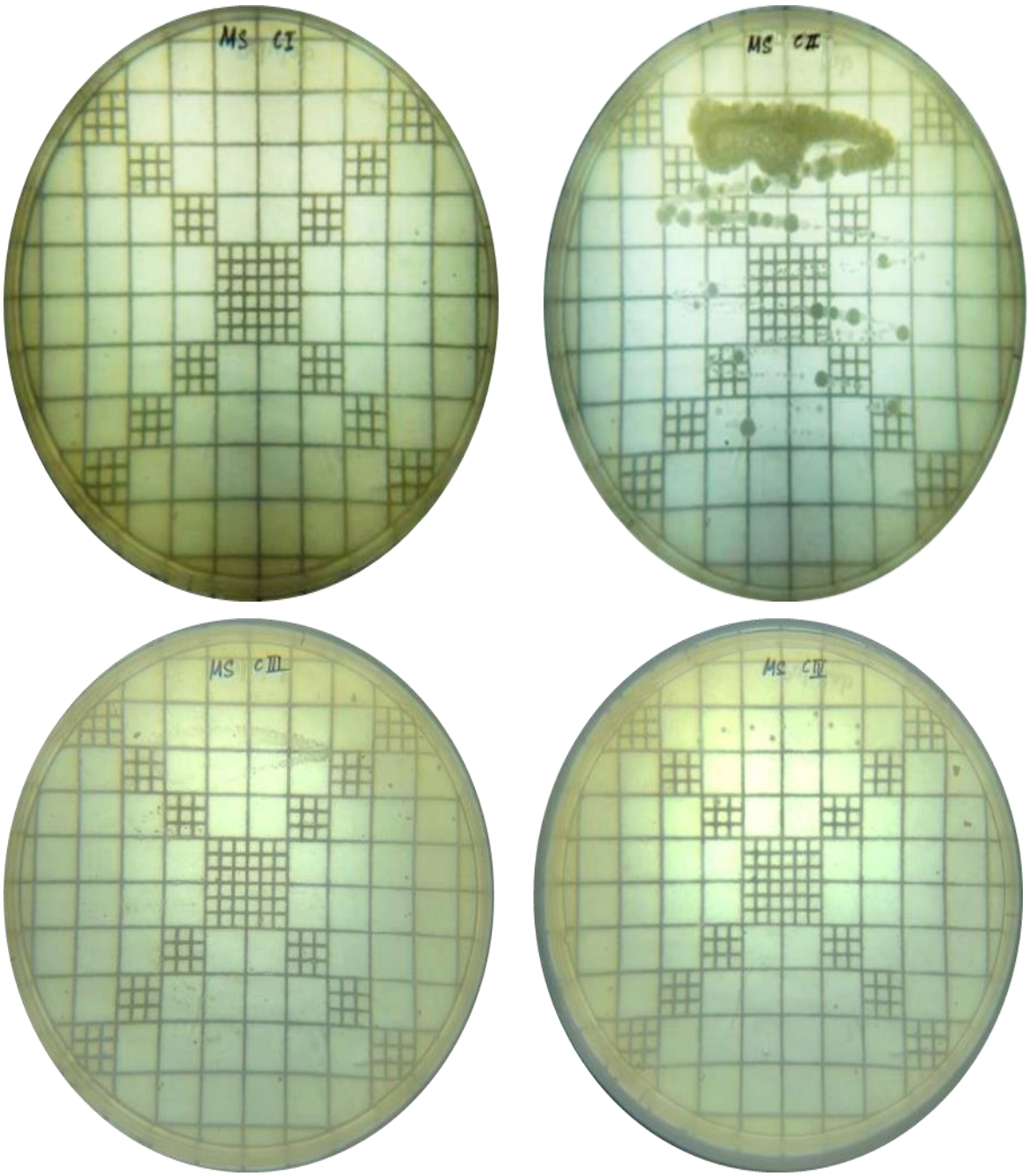

Figure 2: Isolates C1, CI2, CI3 and CI4 inoculated on MS agar plates respectively

ANTIBIOTIC SENSITIVITY TEST OF ANTIBIOTIC BACITRACIN (DISK DIFFUSION METHOD):

Based of the colony characteristics of isolates based on differential culture results, the isolates CI1 And CI2 were tested for their antibacterial resistance against antibiotic bacitracin. The isolate CI1 was observed to show variable resistance to the antibiotic bacitracin (Table 2 & figure 3). Whereas, the isolate CI2, were observed to be susceptible to the antibiotic bacitracin. Zone of inhibition for isolate CI1 was observed to be around 12 mm, which indicates variable resistance against bacitracin.

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Isolates	Zone of inhibition (mm) in Bacitracin containing culture media
CI1	12.04±0.54
CI2	-

Table 2: Antibiotic sensitivity test of CI1 and CI2 growth medium containing antibiotic bacitracin.

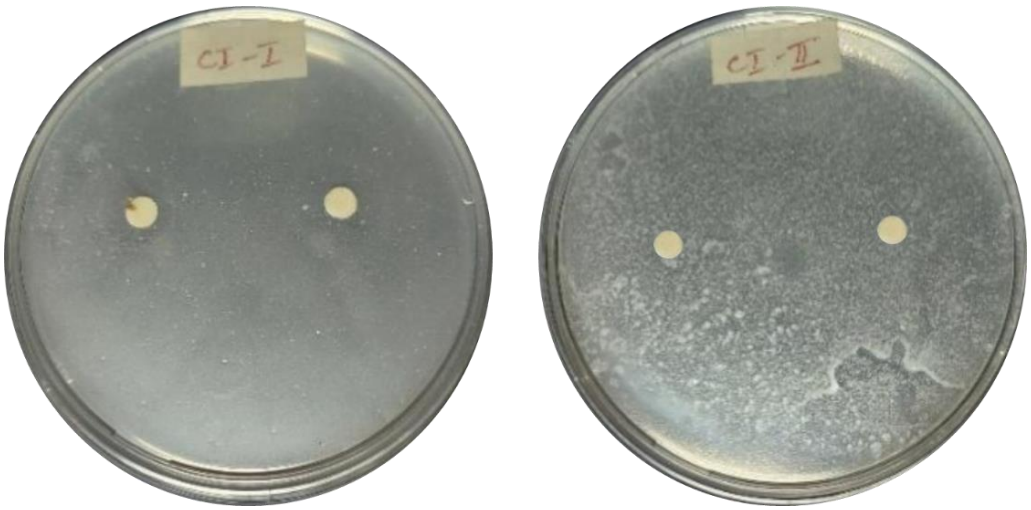
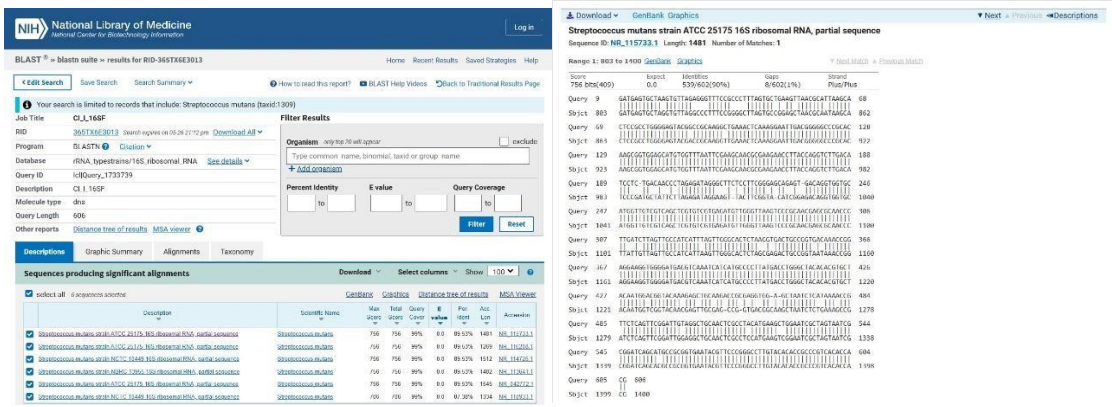


Figure 3: Disk diffusion test of antibiotic bacitracin against culture plates of CI1 and CI2.

16sr RNA SEQUENCING OF THE PURE ISOLATES

All four isolates' genomes were sequencing and the sequences of their 16s RNA were compared to the 16s RNA database in the NCBI website with the help of the BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) tool. Based on the BLAST results, the clinical isolate-I was identified as *Streptococcus mutans* with an E-value of 0.0, percentage identity of 90.0% and query coverage of 99% with database accession NR_115733.1 (Fig 4). The clinical isolate-II was identified as *Limosilactobacillus fermentum* with an E-value of 0.0, percentage identity of 90.0% and query coverage of 99% with database accession NR_104927.1 (Fig 5). The clinical isolate-III was identified as *Veillonella tobetsuensis* with an E-value of 0.0, percentage identity of 96.57% and query coverage of 91% with database accession NR_113570.1(Fig 6) and the clinical isolate-IV was identified as *Actinomyces viscosus* with an E-value of 1e-148, percentage identity of 81% and query coverage of 93% with database accession NR_113030.1 (Fig 7).



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Best hit: Streptococcus mutans strain ATCC 25175 16S ribosomal RNA

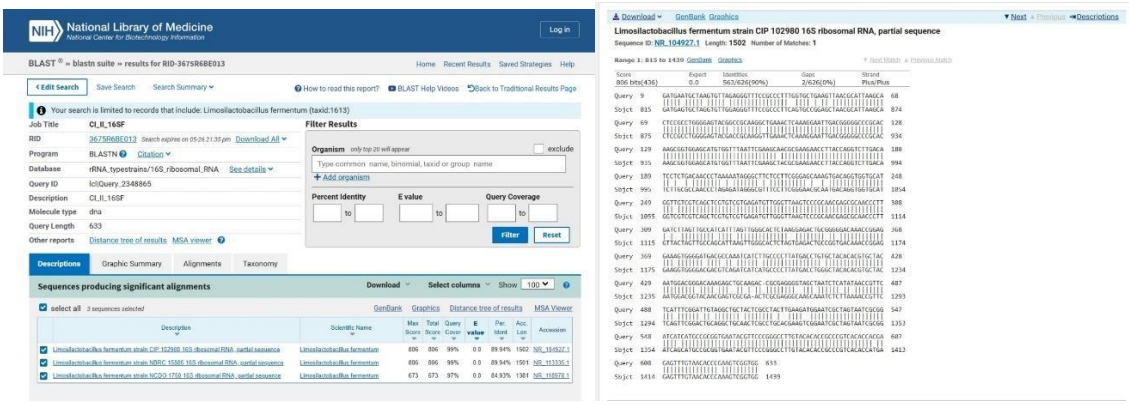
Sequence ID: NR_115733.1

E-value: 0.0

Percentage identity: 90.0%

Query coverage: 99%

Fig 4: BLAST search tool results of isolate CI1



Best hit: *Limosilactobacillus fermentum* strain CIP 102980 16S ribosomal RNA

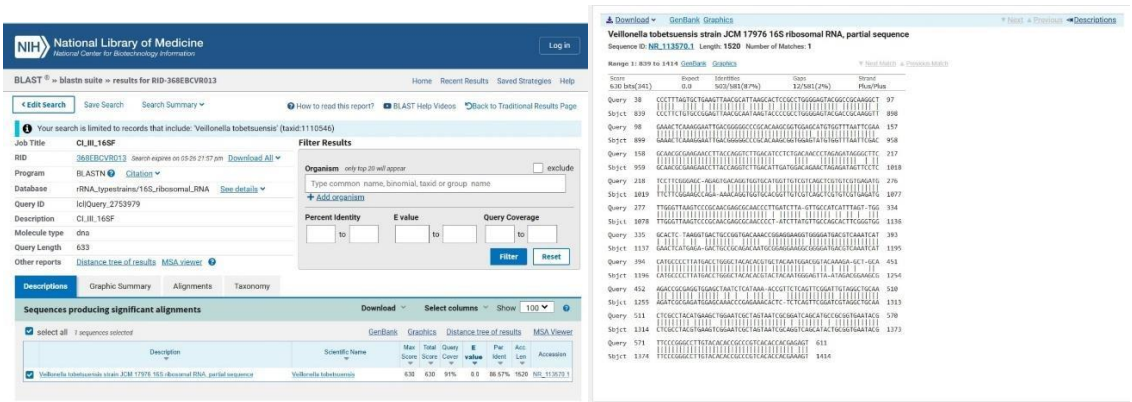
Sequence ID: NR_104927.1

E-value: 0.0

Percentage identity: 90.0%

Query coverage: 99%

Fig 5: BLAST search tool results of isolate CI2



Best hit: *Veillonella tobetsuensis* strain JCM 17976 16S ribosomal RNA

Sequence ID: NR_113570.1

E-value: 0.0

Percentage identity: 91.0%

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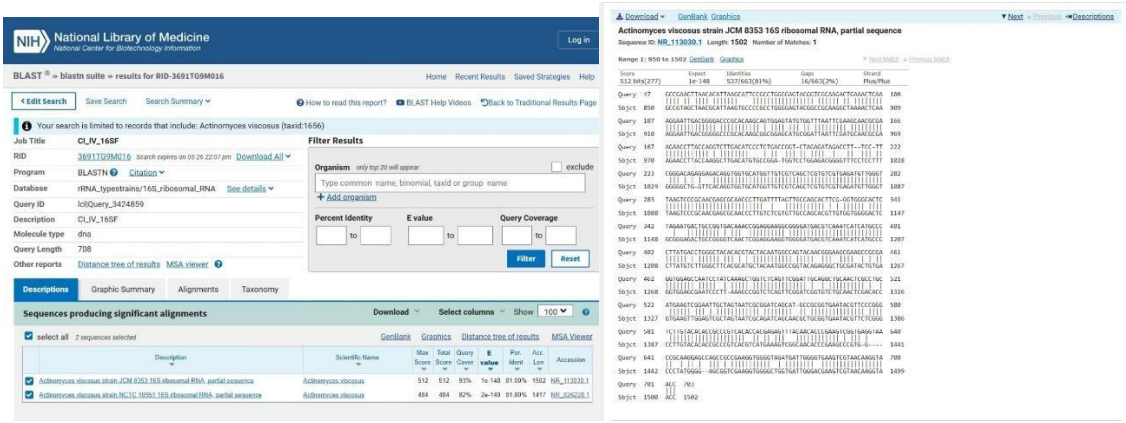
Query coverage: 87%

Fig 6: BLAST search tool results of isolate CI3



Best hit: *Actinomyces viscosus* strain JCM 8353 16S ribosomal RNA

Sequence ID: NR_113030.1

E-value: 1e-148

Percentage identity: 81.0%

Query coverage: 93%

Fig 7: BLAST search tool results of isolate CI4

Discussion:

The oral cavity is similar to other sections of the digestive tracts in having inhabitant micro flora that develops naturally, and which has a distinguishing composition. Due to difference in restricted ecological conditions, the plaque micro flora differs from other mucosal surfaces. The resident micro flora of a site is of benefit to the host by acting as part of host defences by preventing colonization by exogenous species (Rams et al. 1997). The dental plaque is a structurally and functionally organized bio-film. Plaque forms in an ordered way and has a diverse microbial composition. Several bacterial species or cluster of species has been involved in the aetiology of the dental plaque (Bergey and Holt 1994).

The combined cultural and molecular analyses have shown that a diverse community is found in dental plaque and that numerous novel taxa are present (Van Der Waaij et al. 1971). The exposed surfaces of the tooth are to be considered as the open dentin ecosystems. These exposed surfaces are rapidly colonized by microorganisms and covered by dental plaque (Paster et al. 2001). About 1g of dental plaque contains more than 1011 organisms (Meurman 1997). These bacteria remain to the acquired enamel pellicle by specific and non-specific molecular interactions between adhesions on the cell and receptors on the surface (Genco 1992; Gibbons and van Houte 1973). Factors that include in formation of plaque are often affected changes in dietary habits, medication, disease and denture wearing (Beighton et al. 1991). This was also confirmed by Cao et al, (Cao et al. 1990) revealing that diverse ecological factors and concerned in the harbouring of microorganisms and microbial composition on dental plaque. Studies on microbes causing plaque from different geographic regions including developed and developing countries will show variations in the numbers and species of cultivable flora (Aas et al. 2005). The dental plaque is material which supports the growth of wide variety of bacteria. There are almost 500 species of bacteria (Rams et al. 1997). This was in contrast with the finding of Thylstrup A et al (Thylstrup et al. 1987) that, 700 bacterial species were present in the oral cavity, of which only 50 were cultivated and detected.

In the present study, several bacterial species are isolates from the dental plaque and studied. The isolation and

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identification bacteria from dental plaque are based on the appearance of colony and pigment production, staining reactions and biochemical results as well as their molecular parkers. In this current investigation, the isolation and identification is also carried, on the basis of these criteria. Among the isolates Gram positive bacteria were more than Gram negative, which was very much similar to the study conducted here on dental plaque. Streptococcus sp. was more predominant from dental plaque (Pesce et al. 2022; Rozkiewicz et al. 2006; Tada et al. 2006).

In conclusions, culturing of organisms plays a vital role to detect from the dental samples. Antibiotic resistance data required for cultured microorganisms to elucidate of virulence mechanism. However, cultured bacteria may rapidly adjust their phenotypic features in vitro, and 50% of oral microorganism have not till now been cultured (Marsh and Martin 1992; Meurman 1997). It will also be significant to control the oral microflora for systemic reasons since strong associates are being recognized between focal infection of oral origin and a range of systemic diseases as well as coronary heart disease, gastrointestinal disorders and low birth weight, apart from severe overt systemic function. These developments are divided from an improved understanding of the ecological nature of the microbial biofilm that is dental plague, and of its interactions with human host (Newman et al. 1999).

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