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Research Article

16S rRNA Gene Sequence Analysis of Anaerobic Streptococcal Isolates from Patients with Chronic Periodontitis

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ABSTRACT

Periodontal diseases are common oral diseases. Records show high recovery rates of Streptococcus species is associated with untreated periodontitis. Data on variants of streptococcal species associated with periodontitis in Nigeria is limited. This study identified the species, subspecies and genetic relatedness of Streptococcus that occur among patients with chronic periodontitis using 16S rRNA Gene Sequence technique and phylogenetic analysis. Patients were recruited at Periodontology Clinic of Lagos University Teaching Hospital, Lagos Nigeria. Paper point specimens of subgingival plaque were collected from periodontal pockets and cultured by anaerobiosis on Fastidious Anaerobe Agar. Presumptive identification of the streptococcal species isolated was done by cultural and biochemical analysis. Molecular Identification was done by Sanger sequencing to confirm the identity of the isolates. Twenty-four (24) subgingival plaque samples cultured from 6 patients with chronic periodontitis yielded 24 anaerobic gram-positive isolates of which 20 were amplified by I6S rRNA primer. From this, 17/20 (85%) were identified as Streptococcus and 3/20 (15%) Staphylococcus species. The 16S rRNA showed the identity of these isolates at species and subspecies level. The species belonged to the anginosus (58.8%) and mitis (41.2%) groups. Phylogenetic analysis showed divergent periodontal strains. Our results revealed the species and subspecies of members of Streptococcus groups associated with chronic periodontitis in a Nigerian population. The severity of periodontitis in relation to the ecological niche created in periodontal pockets may have an impact on the proliferation of certain Streptococcal species.

Keywords: *Chronic Periodontitis, Anaerobic Streptococcus, 16S rRNA, Sequencing.*

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INTRODUCTION

Periodontitis is the second most common oral condition after dental caries. It is a major health problem and has been linked to some systemic diseases. Chronic periodontitis, a slowly progressive bacterial inflammation of the tooth-supporting tissues is primarily caused by species that are predominantly normal oral commensals. The microbial flora in the subgingival plaque is polymicrobial with considerable genetic variations. These commensals have virulent potentials and the ability to disrupt the normal homeostatic host-microbial interplay in periodontal tissues (Darveau, 2010; Hajishengallis, 2012). The oral aerobic species are dominated by *Streptococcus* species which are the first to colonise the oral cavity after birth. Some species possess ample genetic factors that support their survival in the presence of obligate anaerobes present in deeper periodontal pockets (Richard *et al*., 2014). The genus *Streptococcus* was first described in 1874 by Billrot. Microbes in this group consist of Gram-positive bacteria belonging to the phylum Firmicutes. Their genius is composed of a large variety of commensal and pathogenic species and currently comprises more than 90 species (Teng *et al*., 2014). The technique, 16S ribosomal RNA (16S rRNA) sequencing has enabled the phylogenetic grouping of *Streptococcus* species into eight groups with six groups containing oral *Streptococcus* species (Stackebrandt *et al*., 2002; Richards *et al* 2014; 2017; Abranches *et al*., 2018). The non-oral species are pyogenic and bovis, while the oral species fell into the mitis, sanguinis, anginosus, salivarius, downei, mutans groups (Richards *et al.*, 2014; Gao *et al*., 2014, Abranches, 2018). The oral cavity acts as a reservoir for commensal and pathogenic *Streptococcus* species which can be disseminated to other body sites to cause life-threatening infections (Woo *et al*., 2008; Abranches *et al*., 2018). Species seen as the principal cause of dental caries are occasionally

implicated in subacute infective endocarditis (Woo *et al.*, 2008; Abranches *et al*., 2018). Other conditions are pyogenic non-bacteremic infections (Sunwool and Miller, 2014). bacteraemia (Kobo *et al*., 2017), periodontitis (Kumagai *et al*., 2013), pneumonia (Harai *et al*., 2016), respiratory infections and subacute bacterial endocarditis (Allen *et al.,* 2002) as well as carcinogenesis of the head and neck (Shiga *et al.,* 2001). The *S. anginosus, S. constellatus, S. sinensis. S. intermedius S. sanguinis S. mutans*, and *S. mitis* are involved in one or more types of infection. They may cause opportunistic infections when they find their ways into the bloodstream during invasive procedures such as dental extractions and oral surgery, or daily activities especially mastication, brushing and flossing (Abranches *et al*., 2018).

Advances in molecular studies have revealed the involvement of oral *Streptococcus* species in chronic periodontitis. They form biofilms with reasonable amount of aerobic, microaerophilic and anaerobic oral species making them accessible to every niche of the oral cavity. Defining the taxonomy of the individual specific species and subspecies associated with particular diseases seems to be quite challenging (Velsko *et al*., 2019). In addition to that, oral streptococcal species tend to show resistance to many antimicrobial agents used in therapy (Doern *et al*., 2010; Richards *et al* 2014). The control and management of *Streptococcus* associated infections largely depend on the correct identification of the implicating species or subspecies. Historically, conventional methods such as gram reaction, culture on sheep blood agar, biochemical and serotyping have been used to classify these species. In recent times, molecular methods particularly multilocus sequence analysis, 16S rRNA gene sequencing, and whole-genome are being used to determine taxonomic relationships between the various species. The 16S rRNA gene-targeted sequences, places *Streptococcus* species into their various distinct groups. (Hoshino *et al*., 2005; Richards *et al*., 2014). This molecular technique has given insight into bacterial species inhabiting the oral cavity. In Nigeria, available literature has implicated Streptococcal species in oral infections such as dental caries, periodontal abscess, Ludwig's angina and osteomyelitis of the jaws (Egwari *et al*., 2009), yet there are no sequence data available on 16S - 23S spacer region for any of the *Streptococcus* groups in samples from human oral cavity. Moreover, most reported studies were carried out using cultural methods that may or may not have accurately identified the species or subspecies correctly or were limited to linking only to the genus level. The purpose of this study was to analyse the 16S spacer genes from oral streptococci isolates, in order to establish the species and subspecies harboured in subgingival plaque obtained from patients with chronic periodontitis.

MATERIALS AND METHODS

Study Design and Location: The study was a cross-sectional study conducted at the Periodontology clinic of the Department of Preventive Dentistry, Lagos University Teaching Hospital (LUTH), Idi-Araba, Lagos State. Subjects were selected by convenience sampling from patients with chronic periodontitis who presented to the clinic.

Ethical consideration: Ethical approval was obtained from the Health Research and Ethics Committee (HREC) of the Lagos University Teaching Hospital with assigned number; ADM/DCST/HREC/1944. All subjects gave written informed consent**.**

Participant selection: Chronic periodontitis was determined according to the criteria of the American Academy of Periodontology and the Centre for Disease Prevention and Control (AAP-CDC) Joint Group (Page and Eke, 2007). Patients with systemic conditions such as diabetes mellitus, HIV or cyclical neutropenia, as well as those on antibiotics in the 3 months prior to the commencement of the study were excluded from this study. Current or previous periodontal treatment in the last six months, aggressive periodontitis, endodontically involved teeth and history of smoking were also reasons for exclusion. A full-mouth periodontal examination was conducted on each subject using a dental mouth mirror and UNC-15 periodontal probe.

Collection of subgingival plaque sample: Two sites with probing depths ≤ 3mm (shallow) and ≥ 4 mm (deep) respectively were selected. The teeth and sites were isolated with cotton rolls and the supragingival plaque removed with cotton pellet. Samples were collected with paper points (ISO number 30 UnoDent, England) inserted into the depth of each site for 30 seconds. The paper points were placed immediately into an anaerobic Dental Transport Medium (Anaerobe Systems, USA). Specimens were processed for microbiological culture within 2 hours of sampling. Cultural and biochemical analysis as well as DNA extraction were carried out at the Nigerian Institute of Medical Research (NIMR). While 16S rRNA Sanger sequencing was performed at Inqaba Biotechnical Pty Ltd, Sequencing Facility (Pretoria, South Africa).

Bacterial Isolation and Identification: One sample from each oral site was processed for the identification of *Streptococcus* species as described in Summanen *et al*. (1993). Each sample for bacterial culture was vortexed for 30 seconds and thereafter streaked on Fastidious anaerobic agar (FAA) (Lab M) plate supplemented with 5µg/ml hemin, 1µg/ml vitamin K and 5% of sheep blood. The plates were incubated at 37^{o}C for 3 days in an anaerobic jar (Merck KGaA, Germany). The anaerobic environment was created using 90% nitrite and 10% carbon dioxide generated by sachets of gas generating kit (Merck, Germany) in accordance with manufacturer's instructions. Growth on each plate was examined macroscopically to assess colonial morphology. In order to obtain pure cultures for subsequent and molecular identification, each discrete colony was picked and sub-cultured on Brucella Agar (Oxoid, UK). After 48 hours of incubation, the isolates were Gram-stained and examined under a microscope for cell morphology.

DNA extraction: Each sample for PCR assay was sub-cultured and incubated in Brain Health infusion (BHI) broth (5 ml) for 48 hours and the bacterial cells harvested by centrifugation at 14,000 x g for 10 min. The cells were washed three times in 1 ml of ultra-pure water by centrifuging at

12,000 x g for 5 min before the chromosomal DNA was extracted. DNA extraction was carried out using Fungal/Bacterial DNA MiniPrep™ 50 Preps, (Model D6005). The resulting filtrate was quantified using nanospetrophotomerter Model ND 2000 (Thermo Fisher Scientific, USA) to ensure that adequate amounts of high-quality genomic DNA had been extracted and used as DNA templates for the PCR assays. A gram-negative *E. coli* isolated from same oral sites was used as negative control.

Conventional PCR amplification of 16S rRNA gene: The PCR reaction was performed on the extracted DNA samples using universal degenerate primers combination of 27F (5'AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' TACGG YTA CCT TGT TAC GAC TT 3') that amplifies a 1400 bp 16SrDNA fragment in the variable region. This primer Each PCR reaction contained 5µl of 10 × Taq buffer, 2 mM MgCl 2,1.5 U Super-Therm DNA Polymerase (Southern Cross), 0.25 mM dNTP's, 0.1µM of each primer, 1µl of extracted DNA and Nuclease Free Water (NFW) up to the final reaction volume of 50µl. The PCR cycle started with an initial denaturation step at 94°C for 10 min. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min that was then followed by cooling to 4°C.

Quality control of purified PCR products by Gel electrophoresis: To analyse the amplicon, 10µl of each PCR product was loaded in 1.5% agarose gel in Tris-acetate EDTA (TAE) buffer (40 mM Tris-acetate, 2 mM EDTA [pH 8.3]) and ran at 90 V for 30 min in order to verify amplification. The gel was stained with 0.5 µg/ml of ethidium bromide for 45 min and destained with water for 20 min. Stained gels were examined under ultra-violet (UV) transilluminator in a photo documentation system (Clinix, Model 1500). A DNA ladder digest of 100 bp (Fermenters) was used as a molecular weight marker. The electrophoretic profiles were observed visually for clarity. The gel was observed for presence and absence of bands corresponding to 1400 bp of the 16SrRNA gene targeted. The DNA was recovered from the gel slices using the GeneJET™ gel extraction kit (Fermentas, USA).

DNA Sequencing: DNA Sequencing of the I6SrRNA gene (spa) was performed by Sanger (dideoxy) sequencing technique with an ABI PRISM 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA). Briefly, for sequencing, the thermocycler (A & E Laboratories) was set at an initial denaturation step at 96°C for 20 sec. This was followed by 30 cycles of annealing at 50°C for 20 sec, extension at 60°C for 4 min. Sanger sequencing products were purified using the BigDye X Terminator purification kit (Applied Biosystems). SAM solution (45 ml) and BigDye X Terminator solution (10 ml) were respectively added and premixed in each 0.2ml tube. Thereafter, 5 ml Sanger sequencing product was added in each tube, vortexed for 5 min and centrifuged at 20000 g for 2 min. Then, 10 ml supernatant in each tube was transferred into a plate and covered with septa. After a pulse spin, the plate was mounted in the Genetic Analyzer. The sequences were automatically compiled using Sequencing Analysis 5.3.1 software (Applied Biosystems). The raw DNA sequence traces data was viewed and exported to FASTA formatted text file for BLASTING using FinchTV chromatogram viewer Version 1.4.0 (Geospiza, Inc.).

Bioinformatics analysis: The results were obtained by using nucleotides to determine the nucleotide sequence of the specific microorganism isolated. Sequences obtained were blasted with the NCBI GenBank database against 16S ribosomal RNA Sequences (Bacteria and Archaea) for species or genus assignment (Kim et al 2005; Altschul *et al*., 1997). The highest identity was selected as the identified subspecies, species or genus. For every set of isolate, a read was BLASTED and the resultant top hits with minimum E-score for every BLAST result depicting species name was used to title the specific organism. Edited sequencing result in FASTA format and corresponding identity after BLAST analysis on the NCBI website is as shown in Table 1.

Phylogenetic analysis: To perform phylogenetic analysis of the *Streptococcus* isolates derived. The sequences from the gingival samples were aligned using Clustal Omega (version 1.83) Multiple Sequences Alignment Program. The evolutionary history was inferred using the Neighbour-Joining method in MEGA 6.0 (Saitou and Nei, 1987). Maximum parsimony, neighbour-joining and minimum evolution algorithms were used to create phylogenetic trees.

RESULT

Twenty-four (24) subgingival plaque samples cultured from 6 patients with chronic periodontitis yielded 24 isolates that were gram-positive cocci. The patients were three males and three females aged 33 – 67 with a mean age of 54.7±15.4 years (Table 1). Of the 24 Gram positive cocci isolated 20 (83.3%) were amplified with the 16S rRNA gene and this showed corresponding band while 4 (16.7%) were not. The sequences were trimmed, followed by Chimera Identification for 16S rRNA Sequences (Wright *et al.*, 2012; Murali *et al.* 2018). The *i*dentity of these isolates was revealed at genus, species and subspecies. They were identified as 17/20 (85%) *Streptococcus* and 3/20 (15%) *Staphylococcus* species. The entire length of the 16S rRNA gene sequence of the strains was shown to be 97-98%% identical to species of *Streptococcus.* The sequences were submitted to gene bank and allotted GenBank accession numbers MN559985 - MN559999 as shown on Table 1. The *E. coli* strained isolated, amplified, and sequenced alongside tests strains and used as control was also submitted and allotted MN559989. The *Streptococcus* species isolated from patients with chronic periodontitis belonged to the anginosus (58.8%) and mitis (41.2%) groups respectively (Table 2).

The anginosus groups were mostly *S. anginosus*, and *S. constellatus and S. sinensis*. We also isolated *S. constellatus* subsp. *S. constellatus and S. constellatus* subsp. *pharynges.* The mitis groups were majorly, *S mitis, S sanguinis* and *S. oralis*. The subspecies found in this group were *S. oralis subsp. tigurinus* (Table 2).

T**able 1**
16S rRNA Gene Sequence profile of Anaerobic Streptococcal isolates from Patients with Chronic Periodontitis

Sample No.	Gender	Age (years)	Collection Site (pocket)	Gene bank Species designation	Assigned Genomic Accession Number
PA1	Male	62	Shallow	*Streptococcus sanguinis*	MN559985
PA2			Shallow	*Streptococcus mitis*	MN559986
PA3			Deep	*Staphylococcus epidermidis*	-
PA4			Deep	*Streptococcus oralis* subsp. *tigurinus*	MN559987
PA5	Male	67	Shallow	*Streptococcus anginosus*	MN559988
PA6			Shallow	*Streptococcus constellatus* subsp. *pharynges*	-
PA7			Deep	*Escherichia coli*	MN559989
PA8			Deep	No amplification	-
PA9	Female	65	Shallow	*Streptococcus mitis*	MN559990
PA10			Shallow	*Streptococcus constellatus subsp. pharynges*	MN559991
PA11		37	Deep	No amplification	-
PA12		37	Deep	No amplification	-
PA13	Male	37	Shallow	*Streptococcus constellatus* subsp. *pharyngis*	MN559992
PA14			Shallow_1	*Streptococcus constellatus*	MN559993
			Shallow_2	*Staphylococcus hominis* subsp. *novobiosepticus*	-
PA15		33	Deep	No amplification	-
PA16		33	Deep	*Streptococcus anginosus*	MN559994
PA17	Female	33	Shallow	*Streptococcus mitis*	MN559995
PA18			Deep	*Staphylococcus epidermidis*	-
PA19			Deep	*Streptococcus sanguinis*	MN559996
PA20			Deep	*Streptococcus mitis*	MN559997
PA21	Female	64	Shallow	*Streptococcus sinensis*	-
PA22			Shallow	*Streptococcus sinensis*	MN559998
PA 23			Deep	*Streptococcus sinensis*	-
PA 24			Deep	*Streptococcus constellatus* subsp. *constellatus*	MN559999

Table 2
Percentage distribution of Streptococcus species and subspecies on periodontal sites

Species (Periodontal Site)	*Frequency (N=24)*	*Percentage (%)*
Streptococcus Species	***17***	***70.8***
Anginosus group	**(10)**	58.8
S. anginosus (S)	2	
S. constellatus (D)	1	
S. constellatus,subsp *constellatus* (S,D)	1	
S. constellatus subsp. *pharynges* (S)	3	
S. sinensis (S,D)	3	
Mitis group	**(7)**	41.2
S. sanguinis, (S,D)	2	
S. mitis, (S,D)	4	
Streptococcus oralis subsp. *tigurinus* (D)	1	
Other Species (*Staphylococcus)*	**(3)**	**12.5**
S. epidermis (D)	2	
S. hominis (S)	1	
Non amplified Isolates (D)	**(4)**	**16.7**

Keys: S: Shallow periodontal site, D: Deep periodontal site

The other three (12.4 %) Gram positive cocci were identified by sequencing as *Staphylococcus epidermis and S. hominis* and not *Streptococcus* sp.

Phylogenetic analysis showed divergent periodontal strains associated with chronic periodontitis (Fig. 1). The species isolated fell into two *Streptococcus* groups, that is; anginosus and mitis including members of *sanguinis* group. From this, we observed 6 distinct clusters. Members of Mitis group alone had 4 clusters. Cluster 1 had *S. sanguinis, S. mitis*, and *S. constellatus subsp. pharynges.* Cluster 7 comprised of *S. mitis* alone, while cluster 8 had *Streptococcus oralis subsp. tigurinus and S. mitis respectively.* The fourth cluster of the mitis group was cluster 6 which contains distinct species; *S. sanguinis.* Similarly, cluster 4 and 5 distinguished members of the anginosus group**.** While cluster 4 had *S. anginosus, S. constellatus, S. constellatus subsp. constellatus* and *S. constellatus subsp. pharynges,* the *S. sinensis* alone formed the 5th cluster. The gram-negative *E. coli* species used as a control strain fell into a distinct cluster 2, while all the 3 isolates identified as *Staphylococcus* species were in cluster 3.

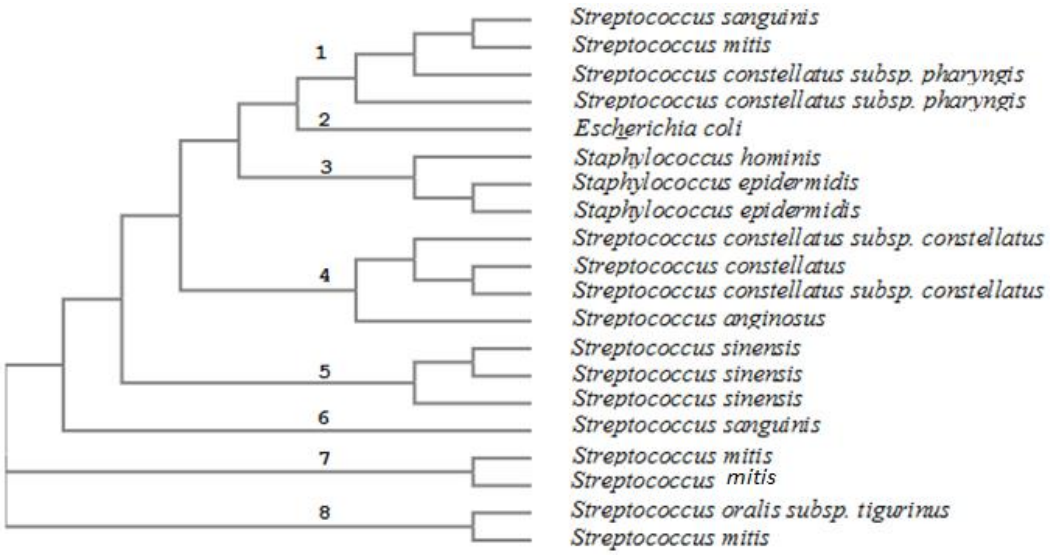

Figure 1:
Dendrogram showing the phylogenetic relationships among sequence of oral streptococci based on 16S rDNA partial genes. The tree was constructed using the neighbor-joining method with Mega 5.02.

DISCUSSION

The 16S rRNA sequencing techniques enable confirmatory identification of bacterial species isolated from clinical specimens, with significantly improved specificity and sensitivity. The study utilized this technique to identify bacterial isolates obtained from periodontal pockets of patients with chronic periodontitis. The presence of Streptococcal species is dependent on the severity of periodontitis and the ecological niche created in periodontal pockets. We observed members of the anginosus, sanguinis and mitis groups in both deep and shallow pockets. None of the isolates obtained belonged to pyogenic, bovis, salivanus and mutants groups. The species and subspecies of anginosus group isolated were *S. anginosus, S. constellatus, S. constellatus* subsp *constellatus, S. constellatus* subsp. *pharynges and S. sinensis*. The pyogenic potentials of species of *Streptococcus anginosus* group are well recognised. While *S. intermedius* is associated with pyogenic non-bacteremic infections, others like *S. anginosus* and *S. constellatus* have been detected in cases of bacteraemia with no abscess or empyema formation (Kobo *et al*., 2017). Increased numbers of both *S. constellatus* and *S. intermedius* are found in periodontitis (Colombo *et al*., 2002; Rams *et al*., 2014). Thus, they are seen to be pathogenic when isolated from humans.

Streptococcus anginosus is a common oral streptococci commensal of the human oral cavity. It is recognized as a causative agent of invasive pyogenic infections (Sunwool and Miller, 2014). The clinical importance is related to its pathogenic involvement in periodontitis, (Kumagai *et al*., 2013) respiratory infections and subacute bacterial endocarditis (Allen *et al.,* 2002) as well as carcinogenesis of the head and neck (Shiga *et al.,* 2001). Furthermore, we obtained *S. sinensis* from both a shallow and a deep periodontal pocket of 10 mm in a 64yr old female diagnosed with severe generalized periodontitis. She had poor oral hygiene with halitosis. Similarly, species and subspecies of *S. constellatus* were detected in our population. This agrees with similar findings (Rams *et al*., 2014). The presence of oral streptococcus in chronic periodontitis interferes with the empirical selection of therapeutic antibiotic agents in patients with positive cultures (Rams *et al*., 2014). Periodontitis involving *S. constellatus* species tends to resist treatment with conventional mechanical root debridement/surgical procedures. It is also known to have variable sensitivity to empirical antibiotics. Although sensitivity patterns were not determined in this study, our result insight into such studies and also portrayed the need to be cautious in selecting therapy, putting into consideration the likely presence of *S. constellatus.* This justifies the need for periodic isolation and subsequent antimicrobial susceptibility testing of oral pathogens to guide therapy.

There is an opinion that species-level identification of strains belonging to the mitis group is challenging due to the large intra and inter-species recombination that exist within the group (Velsko *et al*., 2019). We were able to identify *S. sanguinis* (formerly *S. sanguis*) distinctively. This species falls into the sanguinis group but based on genetic relatedness, they are often known to be included within the mitis group (Zheng *et al*., 2016). Other species from the mitis group identified were *S. mitis* and *S. oralis subsp. tigurinus* species. The species closely related to *S. oralis* especially S. *pneumonia, pseudopneumoniae*, and *S. infantis* are not known pathogens of periodontitis. Thus, any interference in the genomic composition that would have led to overlapping in genomic composition, may have been limited. *Streptococcus oralis subsp. tigurinus* has been isolated from human blood in a condition linked with endocarditis (Jensen *et al*., 2016). The study observed that other oral species like *Streptococcus mutans, Streptococcus gordonii*, and *Streptococcus salivarius* were not isolated. This is not unexpected as they are more or less common oral species implicated in dental caries than in

chronic periodontal conditions (Colombo *et al*., 2002; Rams *et al*., 2014; Abranches *et al*., 2018).

Streptococcus sinensis is described as a causative organism for infective endocarditis in chronic rheumatic heart disease (Teng *et al*., 2014). They were first isolated from blood cultures in 2002 from a Chinese woman with infective endocarditis in Hong Kong (Woo *et al*., 2002), later in 2004 in a similar case (Woo, *et al*., 2004) and in France and Italy (Faibis, *et al*., 2008; Uckay, *et al*., 2007). Although in this study the patient had no history of cardiovascular disease, its presence supports the claim that the oral cavity can be a natural reservoir for *S. sinensis* (Woo *et al*., 2008). According to our phylogenetic analysis using 16S rRNA gene sequences, these species were distinctly located and genetically closely related to anginosus and *sanguinis* and this supports previous findings. (Faibis *et al*., 2008; Uckay, *et al*., 2007; Woo, *et al*., 2008).

Streptococcus sanguinis falls into the sanguinis group of oral streptococci. The species *S. sanguinis* is a common flora in healthy periodontium and less common in periodontitis. (Richards *et al*., 2014). Even with that, their presence in deep (disease) and shallow (healthy) sites of patients with chronic periodontitis is well known. (Ge *et al*., 2013; Kumar *et al*., 2005). This is in line with our study. We also detected the presence of *S. sanguinis* in shallow and deep periodontal sites. There is an opinion that the presence of *S. sanguinis* promotes the reduction of cariogenic *S. mutants* in the oral cavity. In line with this, maintaining a balance of *S. sanguinis* may improve the health of the periodontium. (Abranches *et al*., 2018). Thus, a good probiotic agent to maintain healthy oral microbiome. This, however, needs caution, as these species are genetically distinct, capable of horizontal gene transfer and produce hydrogen peroxide which incite death of neutrophils, thus aiding the survival of bacteria species in the bloodstream (Sumioka *et al*., 2017). Further analysis is required to determine the presence and nature of involvement in deep pockets of patients with chronic periodontitis.

The species *S. mutans* are major microbiota of severe early childhood dental caries but have frequently been isolated in cases of untreated periodontal disease especially in the elderly (Contardo *et al*., 2011). This is not the case with the outcome of our study as none of the isolates obtained were genetically identified as *S. mutans* by 16S rRNA sequencing. *Streptococcus mutans* are considered major pathogens in the initiation and progression of dental caries. They have also been isolated from saliva and subgingival plaque of patients with chronic periodontitis (Dani *et al*., 2016). There is every indication that *S. mutans* thrives in low oxygen tension in deep pockets that favours the growth of microaerophilic species as seen in cases of periodontal disease (De Soete *et al*., 2005). This is also supported by the fact that *S. mutants* possess ample genetic factors that supports their survival in the presence of obligate anaerobes present in deeper periodontal pockets. (Rickard *et al*., 2006). Our inability to isolate *S. mutans* may be due to the choice of fastidious anaerobe agar. Better yield may have been achieved if another specialized medium, like Mitis-salivanus agar was used for primary isolation anaerobically.

Streptococcus sinensis is genetically distinct from other *Streptococcus* groups (Teng *et al*., 2014). They are known to form a cluster close to the *S. anginosus* and *S. mitis* group. The analysis of the dendrogram showed distinct clusters. The species isolated were mainly mitis, *sanguinis* usually grouped as mitis and anginosus streptococcus groups out of which 6 distinct clusters emerged. Four clusters were seen and Cluster 1 had *S. sanguinis, S. mitis,* and *S. constellatus* subsp. *pharynges, The S, sanguinis and S. mitis* are more closely related than with *S. constellatus* subsp. *pharynges.* Cluster 7 comprised of *S. mitis* alone, while cluster 8 had *Streptococcus oralis subsp. tigurinus and S. mitis.* The fourth of mitis group was cluster 6 which contains one distinct species; *S. sanguinis.* Similarly, Cluster 4 and 5 distinguished members of the Anginosus group. Cluster 4 had *S. anginosus, S. constellatus, S. constellatus, S. constellatus and* S. *constellatus* subsp. *pharynges*, while Cluster 5 was only of *S. sinensis* sp. This outcome was not unexpected because it conformed to the phylogenetic pattern emanating from DNA sequencing of Streptococcal 16srRNA (Richards *et al*., 2014; Abranches 2018). Similarly, *S. sinensis* is genetically distinct from other *Streptococcus* group (Teng *et al*., 2014). This was observed, although three isolates were found in both deep and shallow sites of the same patient and fell into a separate group.

Our results revealed the species and subspecies of members of anginosus and mitis groups associated with chronic periodontitis in the studied population. The severity of periodontitis and the ecological niche created in periodontal pockets may have an impact on the proliferation of certain genetically diverse *Streptococcal* species.

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