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A highly efficient computational discrimination among Streptococcal species of periodontitis patients using 16S rRNA amplicons[§]

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Due to the major role played by several species of *Streptococcus* in the etiology of periodontitis, it is important to assess the pattern of *Streptococcus* pathogenic pathways within the infected subgingival pockets using a bacterial specific 16S rRNA fragment. From the total of 50 patients with periodontitis included in the study, only 23 Streptococcal isolates were considered for further analyses, in which their 16S rRNA fragments were amplified and sequenced. Then, a comprehensive phylogenetic tree was constructed and *in silico* prediction was performed for the observed Streptococcal species. The phylogenetic analysis of the subgingival Streptococcal species revealed a high discrimination power of the 16S rRNA fragment to accurately identify three groups of *Streptococcus* on the species level, including *S. salivarius* (14 isolates), *S. anginosus* (5 isolates), and *S. gordonii* (4 isolates). The employment of state-of-art *in silico* tools indicated that each Streptococcal species group was characterized with particular transcription factors that bound exclusively with a different 16S rRNA-based secondary structure. In conclusion, the observed data of the present study provided in-depth insights into the mechanism of each Streptococcal species in its pathogenesis, which differ in each observed group, according to the differences in the 16S rRNA secondary structure it takes, and the consequent binding with its corresponding transcription factors. This study paves the way for further interventions of the *in silico* prediction, with the main conventional *in vitro* microbiota identification to present an interesting insight in terms of the gene expression pattern and the signaling pathway that each pathogenic species follows in the infected subgingival site.

Keywords: *Streptococcus anginosus*, *Streptococcus gordonii*, *Streptococcus salivarius*, *in silico*, periodontitis

The oral cavity is harbored by a complex and diverse microbiota, which comprises hundreds of bacterial species (Paster *et al*., 2001). A symbiotic relationship between the resident oral microbiota and the host is essential for oral homeostasis, while the modification of the subgingival microbiota is particularly involved in the progression of periodontitis (Belstrøm *et al*., 2017). Periodontal disease is a chronic infection of the gums characterized by a loss of the attachment between the tooth and bone, and loss of bone itself (Bansal *et al*., 2014). Periodontal diseases are inflammatory and destructive infections of the dentogingival complex associated with specific periodontal pathogens inhabiting periodontal pockets, which lead to damage of the tooth-supporting tissues such as connective tissue and bone. It may have several negative consequences on the quality of life of the affected individuals, including tooth loss, financial and social problems, and poor alimentation (Popova *et al*., 2013). Although it is generally considered that the periodontal plaque has multifactorial etiology (Al-Jehani *et al*., 2014), data show that some specific Gram-negative microorganisms in the subgingival plaque play a fundamental role in the initiation and progression of periodontitis (Dosseva-Panova *et al*., 2014). Bacteria belonging to the genus *Streptococcus* have gained increasing attention not only because of their

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potential to cause periodontitis (Dani *et al*., 2016). But their coherent presence in the teeth cavity has become a major concern for dentists, since *Streptococcus* plays an important role in teeth cavity infection and treatment (Yadav and Prakash, 2017). Periodontitis is usually caused by the secretions of several varieties of *Streptococcus* in the mouth cavity which potentially leads to a localized breakdown of the dental tissues (Kara *et al*., 2007). Many *Streptococcus* species can induce severe infections of teeth (Robertson and Smith, 2009). A considerable number of researches have focused on several species of *Streptococcus* and their involvement in the periodontitis (Dhotre *et al*., 2018), as their detection in dental biofilm has been considerably employed to predict periodontitis risk and monitor its status (Colombo *et al*., 2016). Also, several studies have recently been developed for the identification of several targeted *Streptococcal* isolates in periodontitis (Marín *et al*., 2016). However, the detection of *Streptococcus* is usually performed using the classical microbiological techniques, which experience multiple drawbacks in terms of the specificity required for the accurate identification of these organisms at the species level. Besides, the currently used biochemical detection techniques lack the presence of a satisfied power to precisely discriminate between closely related species. Therefore, the urgent necessity for the culture-independent techniques is growing at a brisk rate among microorganisms (Al-Shuhaib *et al*., 2018). This diagnosis can be manifested as a direct and rapid picture of the periodontitis-infected teeth, which is considered a mandatory procedure to assess the severity of such pathogenesis. Hence, the intervention of the molecular targeting method including PCR plays an important role in this regard. However, few data are available concerning the behavior of oral streptococci in subgingival samples of patients with periodontitis. On the other hand, the rapid revolution of the modern *in silico* computations may act as powerful tools to provide more information regarding the main pathways each isolated species has taken in its pathogenesis. Furthermore, choosing the conserved 16S rRNA gene over other genes is based on the fact that any particular bacterial variation observed in the site of infection can be directly correlated to the metabolism and virulence properties of these bacteria (Mishra *et al*., 2018). This can be accomplished by combining the conventional PCR-sequencing tool with the state-of-art 16S rRNA-based computational tool to frame an efficient strategy that incorporates the dual ability of the usual *in vitro* detection and the rapid *in silico* characterization. Therefore, this study aims to provide a better insight into the diversity of *Streptococcus* within the periodontitis cases, using the combination of *in vitro* and *in silico* tools.

Material and Methods

Oral specimen's examination

Subgingival samples of 50 patients with periodontitis (20 males and 30 females, mean age: 38 years) were chosen among those requesting dental treatment at the dental clinic of dentistry college, University of Babylon, during the period between January to September 2016. Patients were excluded from this study if they afflicted with other infectious diseases, were pregnant, had a history of previous periodontal medication or if they had received any antibiotic treatment within the past two years of sampling. All patients signed informed consent forms prior to participation. All the procedures used in the present study received prior approval from the committee on the use of human research subjects at the University of Babylon. All admitted participants were itemized in specialized questionnaire papers. A comprehensive dental examination was performed on all the included samples by expert dentists at the same center. The eligible subgingival samples were carefully chosen based one Contardo *et al*. (2011) criteria, including that all participants have at least 20 years old, have not been treated with peritonitis, have at least 25% of teeth in their mouths, have a gingival index of 1 and a hygiene index of lower than 50%. Subgingival samples were collected from the periodontal pocket (3 mm depth) by paper points according to Colombo *et al.* (2009) protocol.

Isolation and identification

All paper point samples were immediately processed in sterile saline and serially diluted 10 folds in 1 ml thioglycollate broth (Code No. CM0173, Oxoid). Fifty µl of diluted subgingival samples were spread on the surface of the plates containing Mitis Salivarius agar (Cat. No. 01337, Sigma-Aldrich). All media were incubated in anaerobic conditions using anaerobic

candle jar for 48 h at 37°C. Subsequently, the recovered colonies on the selective agar media were subjected to various biochemical tests to further confirm their identity, including Gram staining, catalase test, hemolysis in blood agar, arginine hydrolysis and esculin hydrolysis (MacFaddin, 2000). All bacterial samples that were not fully confirmed as *Streptococcus* were omitted from the downstream molecular characterization.

PCR

Streptococci isolates were cultured in LB medium (Cat No. M1245, HiMedia), and incubated at 37°C for 24 h. Genomic DNA was extracted according to the extraction manual of manufacturers (Geneaid). The quantity and quality of the extracted DNA were assessed by a Nanodrop (Biodrop), while its integrity was evaluated by 0.8% agarose gel electrophoresis. The isolated DNA was used as a template for the subsequent polymerase chain reaction (PCR) technique. One ribosomal DNA primer pair that was covering universal consensus sequences in the bacterial organisms was implemented in the present *Streptococcus* spp. amplification. The sequence of the forward primer, 27F, was 5'-GTTTGATCCTGGCTCAG-3', while the sequence of the reverse primer, 1492R, was 5'-AA GGAGGTGATCCAGCC-3' (Kane *et al.*, 1993). The lyophilized primers were purchased from Bioneer Incorporation. The PCR reaction of the PCR primers was performed by Bioneer *Accu*Power PCR premix. Each 20 μl of PCR premix was contained 1 U of Top DNA polymerase, 250 μM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, and 1.5 mM of $MgCl_2$. The reaction mixture was completed with 10 pmol of each primer and 50 ng of genomic DNA. The following program was applied in a gradient PCR thermocycler (Eppendorf). The amplification was begun by initial denaturation at 94°C for 2 min, followed by 33 cycles of denaturation (94°C for 30 sec), annealing (60°C for 30 sec), and elongation (72°C for 30 sec), and was finalized with 3 min of extension at 72°C. subsequently, the specificity of the observed amplicons was verified on 1.5% agarose gel electrophoresis in parallel with a SiZer™ -1,000 plus DNA ladder (Cat No. 24075, Intron Biotechnology).

DNA sequencing

The identity of the observed PCR amplicons was detected by sequencing. All the amplified 16S rRNA amplicons of the 23-microbiologically identified *Streptococcus* isolates were commercially sequenced from both forward and reverse directions using the 27F and 1492R primers, respectively (Macrogen). Only clear chromatographs of the obtained variants were further examined, ensuring that the annotation of the observed variants was not due to sequencing artifacts. The reference sequences of each identified species were retrieved from the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov). The sequencing results of the PCR products were aligned with the respective sequences in the reference database using BioEdit software (ver. 7.1; DNASTAR). Out of 23 Streptococcal isolates that submitted to sequencing reactions, three observed groups of the 16S rRNA variants were deposited in NCBI under accession numbers MK270132, MK270133, and MK270134, which represent *S. anginosus*, *S. gordonii*, and *S. salivarius*, respectively (Supplementary data Table S1). Further details of the observed Streptococcal 16S rRNA variants sequences are also provided by Supplementary data Figs. S1–S3.

Phylogenetic analysis

A comprehensive phylogenetic tree was initially constructed by submitting each observed rRNA variant to NCBI-BLASTn engine (Zhang *et al.*, 2000). The multiple sequences alignments of all obtained variants were combined in one file, the repeated accession numbers were manually omitted and subsequently analyzed altogether by Clustal omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). The Clustal output data were visualized and annotated by Figtree tool (http://tree.bio.ed.ac.uk/software/figtree/). A polar cladogram was built and each incorporated species-group was given a different color within the tree.

Computational analysis

The secondary structures formats of each observed species within *Streptococcus* were constructed by utilizing RNAalifold (Bernhart *et al*., 2008). The output of RNAalifold was retrieved in Vienna format, then, it was subsequently converted by forna server into customizable 2-D structural displays (Kerpedjiev *et al*., 2015). Subsequently, the putative transcription factors

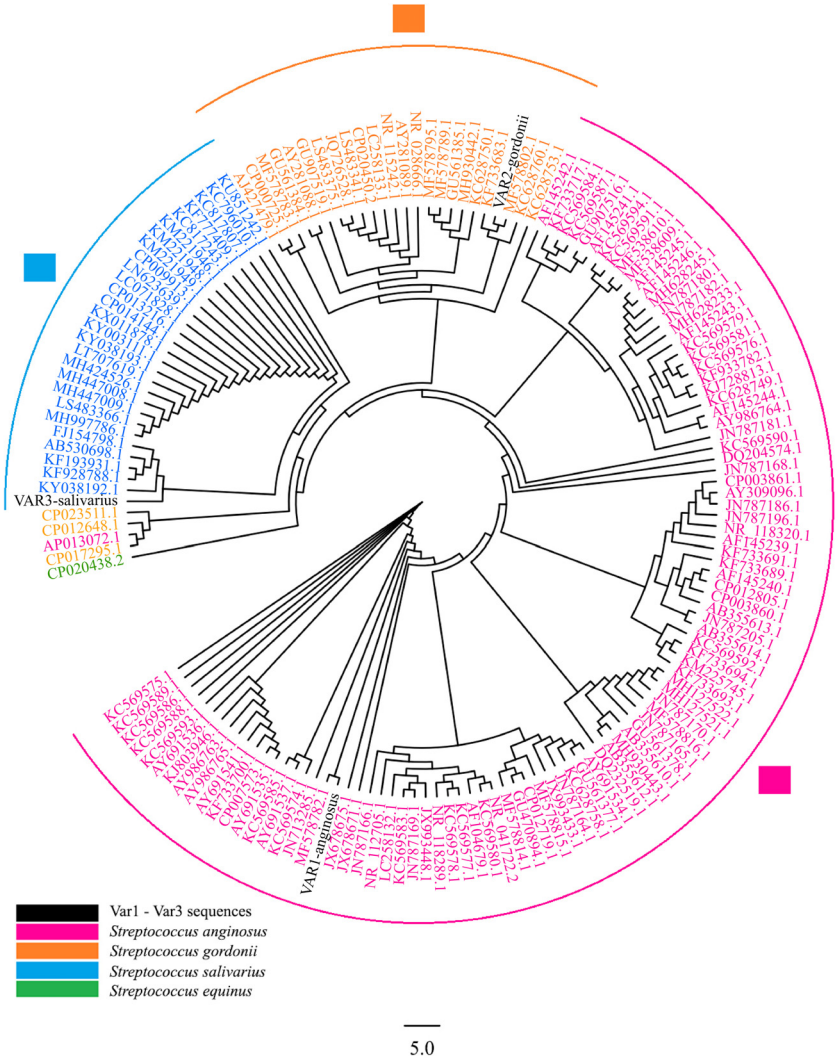

Fig. 1. The comprehensive phylogenetic tree of the 1,492 bp variants of the 16S rRNA gene fragment for Streptococcal stains that isolated from subgingival sites of periodontitis patients. The black color refers to the sequenced three variants, while other colors refer to other referring NCBI deposited species. All the mentioned numbers referred to GenBank acc. no. of each referring species. The number "5.0" at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms.

(TFs) that bind with each constructed 2-D structure were predicted using BPROM software, which predicts the possible TFs binding sites with the recognition sequences (-35 bp) and the unwinding domain (-10 bp) of promoter sequences upstream of the transcription start site (Solovyev and Salamov, 2011). Each point at which the TF binds was highlighted in its corresponding position, and the major differences amongst the highlighted rRNA secondary structures with its corresponding differences were colored appropriately.

Results

A total of identified 53 isolates were tested using several microbiological and biochemical methods. Within the identified bacterial isolates, several genera were recognized, including *Streptococcus*, *Bacillus*, *Lactobacillus*, and *Staphylococcus*. All bacterial strains that were not recognized as *Streptococcus* were not considered in the further study. The remaining 23 subgingival *Streptococcus* isolates constituted about 43.4% of the total bacterial isolates. The amplification of the observed *Streptococcus* isolates had indicated high specificity of the 16S rRNA in terms of providing eligible sequences for downstream

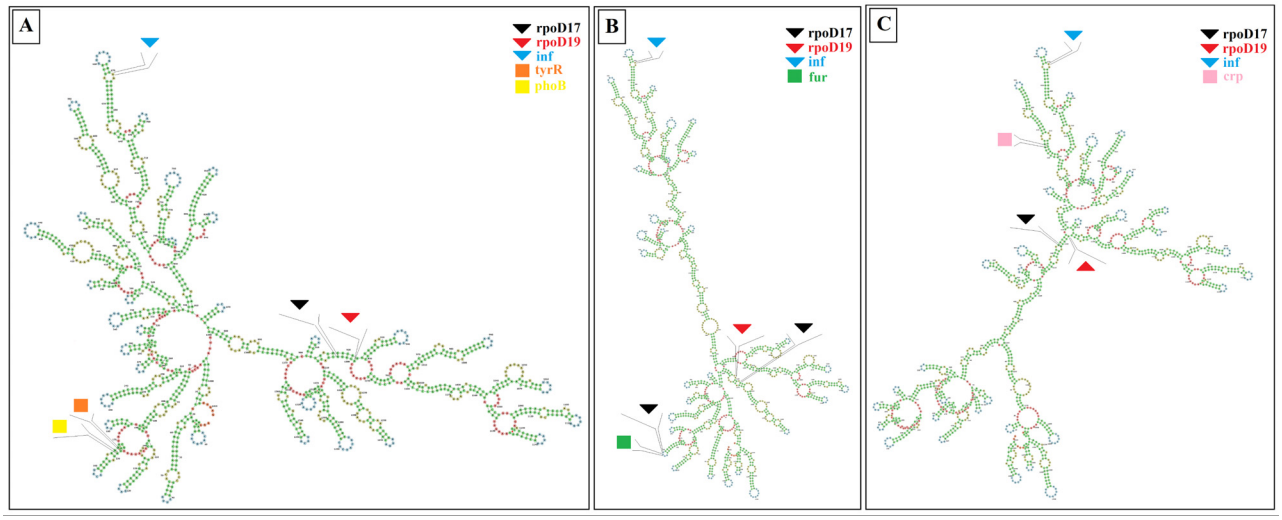

Fig. 2. Putative two-dimensional secondary structures of three16S rRNA variants of three Streptococcal species isolated from subgingival sites of periodontitis patients. All three species characterized by binding with rpoD17, rpoD19, and inf transcription factors. (A) *Streptococcus anginosus* secondary structure that characterized with two exclusive transcription factors, including tyrR, and phoB. (B) *Streptococcus gordonii* secondary structure that characterized with one exclusive transcription factor, including fur. (C) *Streptococcus salivarius* secondary structure that characterized with one exclusive transcription factor, including crp. Each transcription factor is shown in its corresponding binding site in a different color and shape. The secondary structure is built by Forna server, while BPROM server is used to predict the binding sites of transcription factors with their corresponding promoter regions of 16S rRNA variants.

sequencing reactions. Sequencing reactions of 23 subgingival Streptococcal isolates were shown that some of these isolates had little variations with regard to the deposited NCBI Streptococcal database, while the other observed Streptococcal isolates had shown 100% homology with the deposited sequences in the corresponding species within *Streptococcus* genus. However, The NCBI-blastn engine subdivided these isolates into three species groups and it was shown that the most prevalent *Streptococcus* isolates were *S. salivarius* (14 isolates or 60.9%), which was followed by *S. anginosus* (5 isolates or 21.7%) and *S. gordonii* (4 isolates or 17.4%), respectively (Supplementary data Fig. S1).

The currently constructed comprehensive tree indicated the presence of four species allover scanned of 16S rRNA variants. The total number of the aligned nucleic acid sequences in the present 16S rRNA variants-based comprehensive tree was constructed by combining 157 organisms. The comprehensive-tree involved organisms were included *S. anginosus, S. salivairus, S. gordonii,* and *S. equinus* (Fig. 1). It was found that all three observed groups of the 16S rRNA variants of *St. anginosus, S. salivarius*, and *S. gordonii* occupied distinctive positions within the tree. Despite the unique characterization of the studied 16S rRNA variants that originated from several observed variations, no deviation from the corresponding species was noticed among all three groups of variations. This fact was obviously observed in the currently constructed phylogenetic tree as all three Streptococcal groups were accurately positioned without being deviated from their own species. This notion provided a further inclusive indication about the identity of these local studied isolates. Moreover, the accurate determination of each identified species based on the 16S rRNA sequences indicated a noticeable power of the 16S rRNA fragment to efficiently determine the exact identity of each species.

Further in-depth analyses of the nature of the secondary structures of 16S rRNA in each species were provided by combining two types of *in silico* analyses, including RNAalifold and BPROM. This combination was provided a perception for the pattern of secondary structures that were dictated by 16S rRNA sequences and its impact on the binding sites for TF in each specified group. Accordingly, the molecular presentation for the binding of each secondary structure with its corresponding TF was constructed. Three common TFs that were often found in the same sequences of all three recognized Streptococcal species, including rpoD17, rpoD19, and inf (Fig. 2). Four TFs exhibited specific binding sites with their corresponding 16S

rRNA in each species, including tryR, phoB that were only found in *S. anginosus*, while fur and crp TFs were found in *S. gordonii* and *S. salivarius*, respectively.

Discussion

The utilization of the currently available free computational tools improved its efficiency in terms of providing further details of the pattern of each Streptococcal RNA secondary structure as well as its potential preferences to bind with its corresponding TFs. However, several Streptococcal species were not found in the studied subgingival samples. The explanation of this observation varies according to each expected Streptococcal species. For instance, the isolated subgingival samples of this study were not obtained from the deepest subgingival pocket. Thus, 3 mm depth may not be sufficient enough to accommodate plenty of Streptococcal samples (Shi *et al*., 2018). On the other hand, another explanation could be possible, which implies that it is not necessarily mandatory to find such species in several cases of periodontitis patients. This observation was confirmed by the absence of colonization of several pathogenic Streptococcal species in the subgingival positions of periodontitis patients, such as *S. sanguinis*. However, the currently observed Streptococcal species of *salivarius*, *anginosus*, and *gordonii* were usually available in such subgingival positions. With regard to *S. salivarius*, it was reported that this species was one of the pathogenic organisms that usually localized in such infected oral regions (Stingu *et al*., 2008), while other reports indicated a protective role of this bacteria against oral pathogenesis (Moon *et al*., 2016). With respect to *S. gordonii*, the pathological situation was different in this species as this organism was infrequently associated with invasive infections (Dadon *et al*., 2017). However, an exclusive clinical presence of *S. gordonii* in periodontitis patients was also reported (Stingu *et al*., 2008). However, it was demonstrated that *S. gordonii* plays a particular role in the early colonization with *Porphyromonas gingivalis* (Berezow and Darveau, 2011), whilst another study has associated this species with healthy subgingival sites (Haffajee *et al.*, 2006). On the other hand, the comprehensive tree that built on 16S rRNA amplicon was indicated high accuracy of this fragment to give extremely powerful discrimination among three observed subgingival *Streptococcus* species. Although high numbers of resolved microorganisms were involved in this tree, all the incorporated organisms were found to belong to the same species. Thus, it is rational to say that the 16S rRNA-based tree is of high specificity. However, each observed subgingival species variant showed a particular 16S rRNA secondary structure within the comprehensive tree. Therefore, each rRNA structure order certain TFs to bind to its sequences-dictated secondary structure. In particular, when the microbial pathogen encounters variable circumstances during the natural course of infection alternative sigma factors have been shown to regulate the expression of genes in many cases. Furthermore, the RpoD sigma factors have been shown to be important for virulence in the observed Streptococcal species (Wilson *et al*., 2002). The conserved nature of rpoD TFs in the currently observed three Streptococcal groups indicates the fact that the diversity of 16S rRNA is potentially not related to this type of TF. This finding may suggest no intervention of rpoD sigma factor in altering the pathogenesis route followed by the observed three Streptococcal groups. However, *S. anginosus* offers binding sites for phoB and TyrR TFs. The phoB TF was implicated in the crosstalk with other regulatory proteins involved in virulence, antibiotic resistance, and biofilm formation (Bachhawat *et al*., 2005), while TyrR TF was regulating the expression of the bacterial genes involved in the uptake and metabolism of aromatic amino acids (Coulson and Patten, 2015). Thus, the alteration in both phoB and TyrR TFs among the studied *Streptococcus* species might be reflected in a dramatic change of the mechanism through which *S. anginosus* reacts with the infected subgingival sites and which differs from the other two observed *Streptococcus* species. With regard to *S. salivarius*, it was characterized by the binding with one of the most prolific TF in bacteria, crp (cAMP receptor protein) or cap (catabolite activating protein), which was activated in response to glucose starvation and other stresses (Seshasayee *et al*., 2011). It is a trans-acting activator that plays an important role in catabolite repression (Lawson *et al*., 2004), while *S. gordonii* isolates were characterized with the exclusive presence of the fur (ferric acid uptake regulator) TF, which was involved in the contribution of the virulence of invading bacterial species by controlling the transport of several

important minerals (Troxell and Hassan, 2013). Therefore, the fur-involved alteration would remarkably substitute the corresponding 16S rRNA sequences in *S. salivarius* species as it was predicted computationally in the present work. Thus, our study has important implications for the epidemiology of *Streptococcus* in periodontitis, as *Streptococcus mutans* is directly associated with the development of dental caries, while less acid-tolerant behavior has been found in the other two species of *Streptococcus salivarius* and *Streptococcus gordonii*, since they produce large amounts of alkali displaying an important role in the acid-base physiology of the oral cavity (Abranches *et al*., 2018). These differences in the site of infection have been highlighted by constructing the 16S rRNA-based groups. These species-specific secondary structures have shown different profiles in terms of binding with TF in each case, suggesting that at least three mechanisms of Streptococcal pathogenesis. Thus, this 16S rRNA-based prediction provides important information about the sort of Streptococcal nucleic acid variations and suggests free computational strategies to illustrate the molecular mechanisms of this pathogenesis; therefore, it is more likely to get implemented in diagnostic laboratories within a short timeframe.

Conclusion

Form the above-stated results, it would be rational to conclude that each observed *Streptococcus* species occupies a particular secondary structure of the 16S rRNA. This sequence-dictated structure would order different TFs to bind specifically to each observed Streptococcal species. These binding differences would presumably have a certain manifestation on a variety of differences in the dealing with the site of infection. Therefore, the combination between *in vitro* PCR-sequencing strategy, the *in silico* built 2-D structures, and their predictive TFs would provide an in-depth insight into the nature of pathways through which each Streptococcal species follows in its pathogenesis in the infected periodontitis site.

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