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Effect of High Altitude on Oral Bacterial Biodiversity and Immune Response in Saudi Adolescent Males with Periodontitis, Asser Region Southwestern of Saudi Arabia

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Abstract: Salivary samples were collected from 200 Saudi adolescents with periodontal disease (gingivitis, periodontitis) from Abha city, Asser region, southwestern Saudi Arabia (with an altitude of 3,200 meters above the sea level) with low oxygen tension. Aerobic bacteria were isolated on broth medium (Lb). Hundred bacterial isolates were purified and examined morphologically and by Gram stain. Thirty bacterial isolates were selected based on the colony morphology and Gram staining. These isolates were fingerprinted using five different arbitrary primers. The RAPD-PCR succeeded to group the examined 30 bacterial isolates into 6 different groups; each group consisted of many subgroups. Some bacterial isolates conservative to each group were selected and the 16S rRNA gene for each was amplified and sequenced. Sequence analysis revealed that *Streptococcus mutans*, *Porphyromonas gingivalis* (Gram–ve), *Staphylococcus epidermidis* (Gram positive), *Streptococcus salivarius* (Gram positive) *and Lactobacillus sp* (Gram positive) are the predominant organisms in the examined persons. For studying the defense system response against the five different species excited in the Saudi adolescents, DNA was isolated from saliva and the extracted DNA was examined with four different defensin genes. Different PCR amplicons were observed and sequenced and the sequence analysis revealed that, all the obtained nucleotide sequence belongs to the beta defensin genes with different percentage of similarity. The amplified defense genes from different salivary samples showed the same structure between the examined communities but showed low similarities with the others defensins presented on data base. However the low oxygen tension characterizing this area of the world may have a potential effect on the oral bacterial biodiversity and host immune response.

Key words: Oral bacteria · Immune response · RAPD-PCR and Defensin genes

INTRODUCTION

In oral microbiology it is essential to identify and characterize the various microorganisms involved in the different oral infections and such information will be useful for monitoring the disease progression [1]. It is known that periodontal pockets can harbor more than 300 different bacterial species [2, 3]. Organisms such as *Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia or Eikenella corrodens* have been considered to have a role in periodontal disease [3, 4]. However, most of these organisms can also cause serious non oral infections [5]. Several studies have demonstrated a strong correlation between the presence of putative periodontal organisms and the destruction of periodontal tissues [4, 6].

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Different methods of detecting phenotypic or genotypic variations have been used to characterize periodontal pathogens and differentiate them from commensal strains [6]. Molecular methods are currently available for the identification and subtyping of bacterial strains such as *A. actinomycetemcomitans* and *P. gingivalis*, but they vary in efficiency and the amount of required labor [7, 8].

There is evidence indicating that *P. gingivalis* may be a keystone species in the oral microbial community. Keystone species in this context means that this one bacterium serves an essential function for the entire community, similar to a differentiated cell serving a function for an entire tissue. The presence of the *P. gingivalis* TLR4 lipid A antagonist that can block TLR4 activation in response to several different oral microbial bacteria [9] through competitive binding to MD-2 [10, 11] combined with the observation that *P. gingivalis* releases LPS that can penetrate gingival tissue [12] supports the notion that the TLR4 lipid A antagonist will dampen TLR4 responses for the entire oral microbial community. This is especially relevant considering the proposal by Munford and Varley [13] that TLR4 sensing prevents invasion into submucosal tissue by mucosal Gram-negative bacteria. Therefore, heam may act as an environmental sensor that *P. gingivalis* responds to by making the lipid A TLR4 antagonist and then this facilitates invasion of tissue and modulation of innate host defense mediator expression in response to numerous members of the oral microbial consortium. Techniques used to identify putative anaerobic pathogens from destructive periodontal disease such as bacterial culture is somewhat cumbersome and expensive [14]. Similar to the association found between the commensal oral microbial flora and the innate host defense status of clinically healthy tissue, there is a strong correlation between the microbial flora and a destructive inflammatory response [15]. Recently, it has been demonstrated that clinically healthy tissue displays low level expression of select inflammatory mediators. The expression of E selection on the vascular endothelium, for example, is believed to facilitate leukocyte exit from the vasculature into surrounding tissue where they remove bacteria [15].

Aseer region extends from the high mountains of Sarawat (with an altitude of 3,200 meters above the sea level) to the Red Sea, with low oxygen tension which may have a role affecting oral microflora and host defense mechanism. So, this work aimed to study effect of high altitude with low oxygen tension on the oral bacterial biodiversity in Saudi adolescents with periodontal disease as well as defense of those patients, in Asser region.

MATERIALS AND METHODS

Salivary Sample Collection: We collected 5-min paraffin wax-stimulated saliva samples from Saudi adolescent male volunteers from different schools in Asser region, Saudi Arabia after written consent from their parents. Two hundred persons with periodontal disease and 100 free from any periodontal disease as control. All individuals recruited for this study were systemically free, not under any kind of medications, non smokers or had any other habits which may affect oral bacteria (Qhat chewing habit, Shamma, Snuff, Timbak). Clinical oral examination using disposable diagnostic kits and periapical x-ray radiograph were used to diagnose periodontis and gingivitis.

Bacterial Isolation from the Collected Saliva: Collected salivary samples were inoculated in broth medium (LB) and aliquots were spread onto Petri dishes containing LB agar medium. Pure colonies were selected and streaked into new plates. Microscopic examination and Gram staining were performed for all the obtained bacteria to distinguish between them (16).

Genotypic Characterization of Bacterial Isolates

DNA Extraction Protocol: Total DNA was extracted for 30 bacterial isolates according to the procedure carried out by stock *et al*. [16].

Random Amplified Polymorphic DNA (RAPD) Analysis: Five 20–mer arbitrary primers were used in RAPD analysis. Sequences of primers are illustrated in Table (1). For RAPD analysis, PCR amplifications were carried out in a total volume 25 µl containing 2.5 µl 10 x buffer, 2 µl 25 mM MgCl2, 2 µl 2.5 mM dNTPs, 1 µl 10 pmol primer, 1 µl 50 ng of genomic DNA and 0.2 µl Taq DNA

Table 1: Sequences of primers used in RAPD analysis

Primer	Nucleotide sequence 5\ to 3\
A1A13	CAGGCCCTTCCAGCACCCAC
A9B7	GGTGACGCAGGGGTAACGCC
RAPD9	GGT CTA CAC C
Rpo4	GGA AGT CGC C
A1	CGAGCCCTTCCAGCACCCAC

Table 2: Defensin genes primers used in this study

Primer name	Nucleotide sequence	Annealing Temp.
HBD1	'5- CCCAGTTCCTGAAATCCTGA- 3'	
	'5- CAGGTGCCTTGAATTTTGGT- 3'	58°C
HBD2	‘5- CCAGCCATCAGCCATGAGGGT-3’	
	‘5- GGAGCCCTTTCTGAATCCGCA-3’	61°C
HBD3	'5-ATGAGGATCCATTATCTTCT-3'	
	'5-TTATTTCTTTCTTCGGCAGC-3'	62°C
HBD4	'5-TGTTTGCTTTGCTCTTCCTG-3'	
	'5-CTTTCTTCGGCAGCATTTTC-3'	57°C

polymerase (5 units/µl). PCR amplification was performed in a thermal cycler (Eppendorf, company, USA) programmed for one cycle at 95 0C for 5 min. Then 34 cycles were performed as follows: 30 s at 95°C for denaturation, 1 min at 45°C for annealing and 2 min at 72°C for elongation. Reaction was then incubated at 72°C for 10 min for final extension.

Electrophoresis was performed at 100 Volt with 1 x TBE buffer in 1.5% agarose/1 x TBE gels and then the gel was stained in 0.5 µg/ml (w/v) ethidium bromide solutions and destained in deionized water [17].

PCR Amplification for the 16S rRNA gene for the Different Bacteria: Primers 27F and 1492R, corresponding to the positions 7-27 and 1492-1509 bp respectively [18, 19] of the *E. coli* 16S rRNA conserved gene sequence, were used to amplify approximately the full length of this gene in rhizobia. Primers were supplied by MWG, Germany. PCR amplifications were carried out in a total volume 25 µl containing 2.5 µl 10 x buffer, 2 µl 25 mM $MgCl_2$, 2 µl 2.5 mM dNTPs, 1 µl 10 pmol of each 27F and 1492R primers (Table 2), 1 µl 50 ng of genomic DNA and 0.2 µl Taq DNA polymerase (5 units/µl). PCR amplification was performed in a thermal cycler (Eppendorf company, USA) programmed for one cycle at 95°C for 5 min. Then 34 cycles were performed as follows: 1 min at 95°C for denaturation, 1 min at 55°C for annealing and 2 min at 72°C for elongation. Reaction was then incubated at 72°C for 10 min for final extension and stored at 4°C.

DNA Sequencing and Sequence Analysis of 16S r RNA Amplicons: The amplified PCR amplicons of the 16S r RNA gene (1500bp) were cut from the agarose gel and purified using the agarose DNA purification kit (Qiagen, Germany). The purified fragments were subjected to DNA sequencing using the automated DNA sequencer (Macrogen Company, Korea). The sequence analysis was performed using NCBI database and the obtained DNA nucleotide sequences were submitted to gene bank to get the accession numbers.

Amplification of the Defensin Genes in Saliva of One of the Examined Persons: To study the response of the immune system on the saliva of one of the randomly selected students whose saliva contained five bacterial species.

DNA Extraction: DNA was extracted from the child saliva using blood and fluid DNA extraction kit (Qiagen, Germany).

Amplification of Defensin Genes from the Extracted DNA: The genomic DNA was subjected to PCR amplification using four different beta defensin genes primers [20] (Table 2). The PCR reaction was performed in total volume 25ul and the reaction constituents were as previously described. The reaction conditions were as following; initial denaturating step at 95°C for 5 min followed by 34 cycle of denaturating at 94°C for 1 min, annealing at (58, 61, 62 and 57°C) for 1min and extension at 72°C for 1 min, followed by 10 min at 72°C for elongation. The PCR reaction was then stored at 4°C until used.

DNA Sequence and the Sequence Analysis of the Amplified Defensin Genes: The amplified PCR amplicons of defensin genes were cut from the agarose gel and purified using the agarose DNA purification kit (Qiagen, Germany). The purified fragments were subjected to DNA sequencing using the automated DNA sequencer (Macrogen Company, Korea). The sequence analysis was performed using NCBI database and the obtained DNA nucleotide sequences were submitted to gene bank to get the accession numbers.

RESULTS

RAPD-PCR and Bacterial Fingerprinting: More than 100 hundred bacterial isolates were isolated from the saliva of the Saudi adolescent males, Asser region.

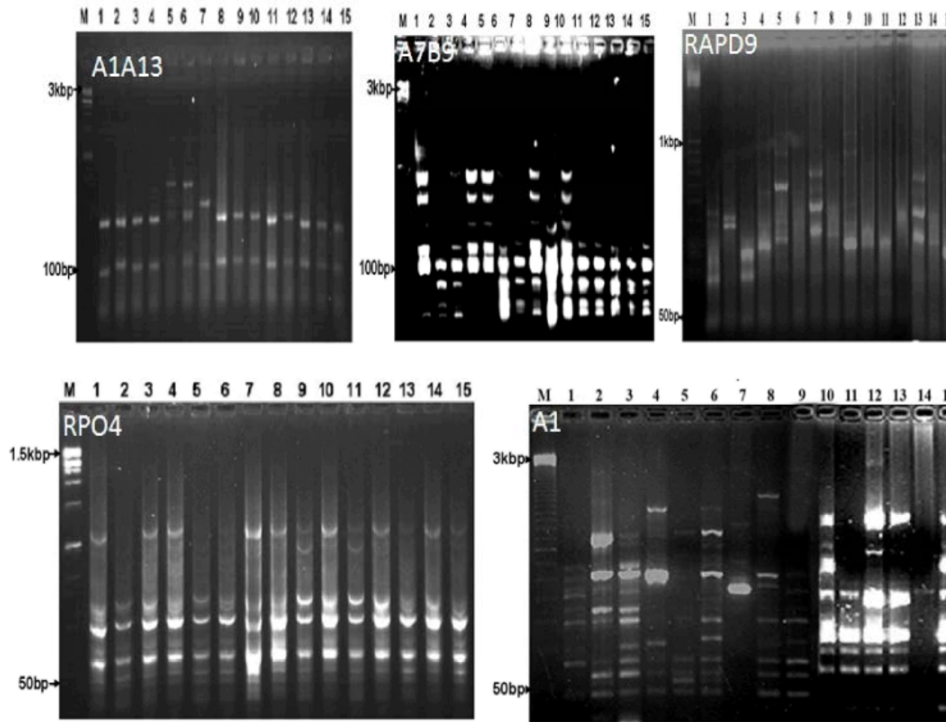

Fig. 1: RAPD-PCR for the first examined 15 bacterial isolates using five different arbitrary primers. Primer name indicate at the right corner of the picture. Lanes; M: DNA marker 1500bp. Lanes from 1 to 15 the isolated bacteria from the human saliva.

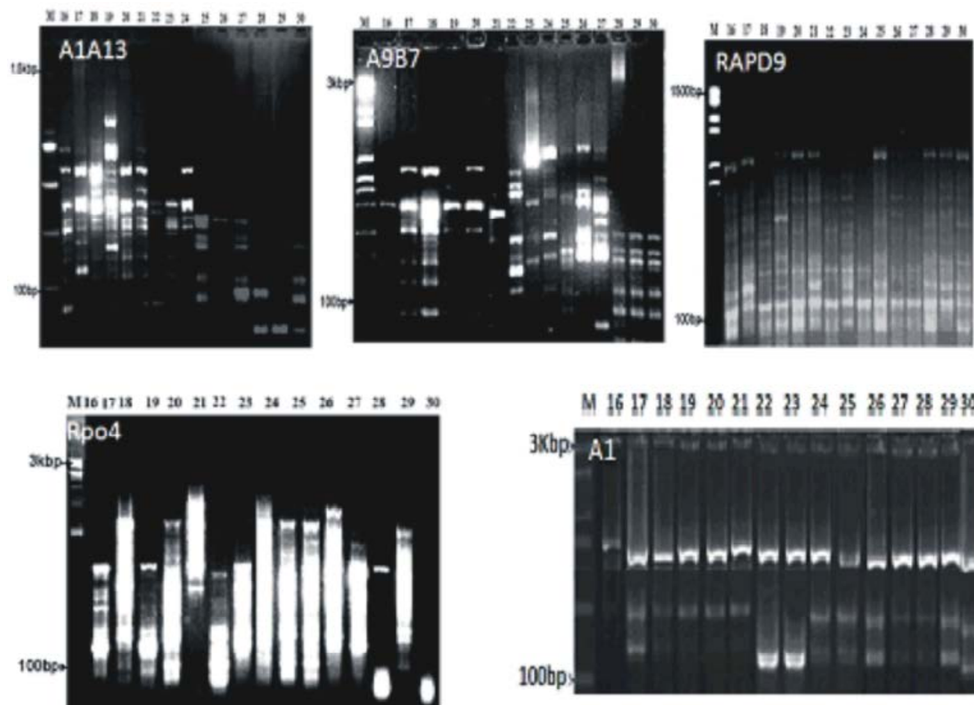

Fig. 2: RAPD-PCR for the second examined 15 bacterial isolates using five different arbitrary primers. Primer name indicate at the right corner of the picture. Lanes; M: DNA marker 1500bp. Lanes from 1 to 15 the isolated bacteria from the human saliva.

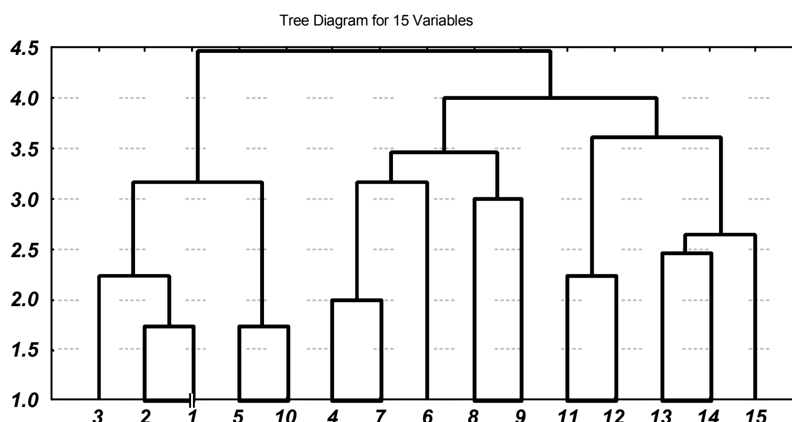

Fig. 3A: Dendogram for the first examined 15 bacterial isolates based on the band patterns obtained by the five arbitrary primers

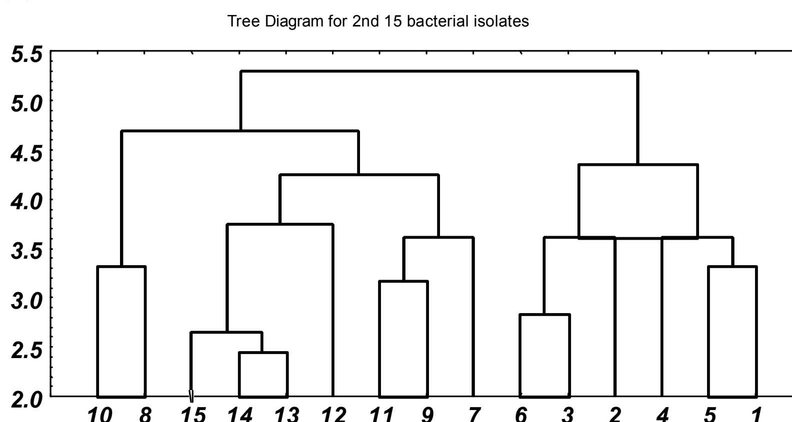

Fig. 3B: Dendogram for the 2nd examined 15 bacterial isolates based on the band patterns obtained by the five arbitrary primers.

The bacterial isolates were screened based on the colony size and the morphology. The results showed that about 30 isolates were differed (data not shown) among the 100 cultured isolates. The bacterial cells were divided into two groups each group contained 15 bacterial isolates. The 30 bacterial isolates were subjected to RAPD-PCR using five different arbitrary primers as shown in Table 1. Different band patterns were obtained with each isolate. Bands with different molecular weights ranged between 3kbp to 100 bp were observed.

The data presented in figure 1 showed that, primer A1A13 succeeded to give only 4 different band patterns two of them were monomorphic bands. In case of the primer A9B7 the 15 examined isolates gave different pattern (7 patterns) all of them were polymorphic. The molecular weight of the band patterns ranged between 2kbp to 100bp as shown in Figure (2).

Primer RAPD9 showed different band patterns; four different bands with molecular sizes ranged from 600bp to 50bp. On the other hand primer Rpo4 gave five different band patterns with molecular sizes ranged from 500bp to 50bp. Finally primer A1 succeeded to differentiate between the examined 15 bacterial isolates and gave different band patterns (18 band patterns) and all the obtained band patterns were polymorphic bands.

In case of the other 15 bacterial isolates (Fig. 2); primer A1A13, about 15 different band patterns were obtained. The molecular weights of the obtained band patterns ranged from 1kbp to 50bp. Whenever, primer A9B7 succeeded to differentiate between the examined 15 bacterial isolates and gave 14 different band patterns. The molecular sizes of the obtained band patterns ranged from 1.5kbp to 50 bp. Primer RAPD9 gave different band patterns (8 patterns) all of them were polymorphic. The molecular sizes ranged from 1 kbp to 50bp. In case of primer A1 about 6 band patterns with molecular weight ranged from 1 kbp to 50 bp. All the obtained bands were polymorphic.

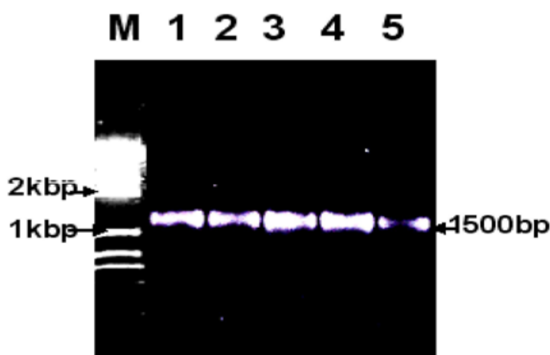

Fig. 4: 1.5 kb amplified product of 16S rRNA gene for the five selected isolates

When the dendogram was constructed based on the obtained band patterns for the thirty examined bacteria, results presented in figure (3A &3B) revealed that, the five arbitrary primers divided the first 15 bacterial isolates to three different groups. On the other hand the same five primers divided the second 15 bacterial isolates into two main groups. Based on the obtained five different groups we selected one isolate to be representative for each group to be sequenced.

Bacterial Identification Using 16S rRNA Gene and Their Accession Numbers: The bacterial DNA was subjected to 16S rRNA amplification and amplicon with 1500bp was obtained. The purified DNA amplicon was sent to Macrogene Company (Korea) for sequencing. The DNA sequence presented in figure (4) revealed that the five selected bacterial isolates were; Streptococcus mutans, Porphyromonas gingivalis (Gram –ve), *Staphylococcus epidermidis* (Gram positive), *Streptococcus salivarius* (Gram positive) and *Lactobacillus sp* (Gram positive). The isolates were similar with the other related bacteria on Gene Bank with similarity ranged from 99 to 100%.

Sequencing and Sequence Analysis of the Selected Isolates: The nucleotide sequence was submitted to the Gene Bank and the obtained accession numbers were listed as shown in Table (3). The phylogeny tree (Fig. 5) was constructed based on the obtained DNA nucleotide sequence of the 16S rRNA genes, the results revealed that five bacterial isolates were divided into two main groups. Group one contained the two streptococcus isolates, lactobacillus and staphylococcus. Whenever, the second group contained only the Prophyromonas isolate.

Amplification of 4 Different Defensin Genes from Human Saliva: The results indicated that, with primer HBD1 there were two amplicons at position 1000 and 179bp, with primer HBD2 there were three amplicons at position 800, 215 and 179bp, with primer HBD3 there were two amplicons at position 205 and 450bp and with primer HBD4 there were two amplicons at position 257 and 600bp (Fig. 6).

using three different sets of primers; HDB1, HDB2, HDB3 and HDB4. M2 and M1 are ladder DNA markers 1kbp and 10kbp respectively.

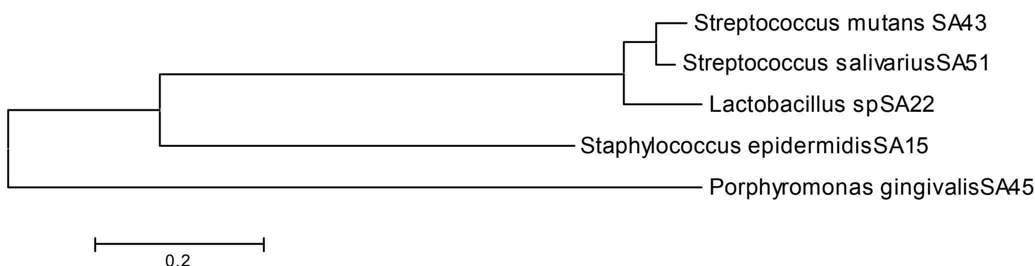

Fig. 5: Phylogenic tree for the nucleotide sequence of the 16S rRNA of the five different bacterial isolates. The phylogeny was constructed by the program Mega 4.

Table 3: The Gen- Bank Accession numbers of the five selected bacteria

NO.	Name of the organism	Accession no. in GenBank
1	*Streptococcus mutans* SA43	JQ894504
2	*Porphyromonas gingivalis* AS45	JQ894505
3	*Staphylococcus epidermidi* SA15	JQ894506
4	*Streptococcus salivarius* SA51	JQ894507
5	*Lactobacillus sp* SA22	JQ894508

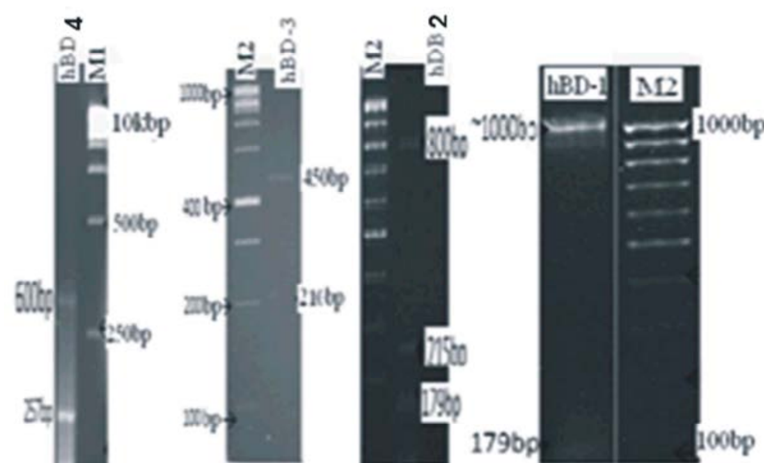

Fig. 6: PCR amplification for three different human beta- defensin genes amplified from the human saliva

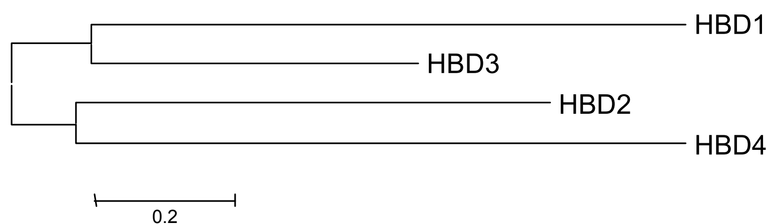

Fig. 7: Phylogenic tree for the nucleotide sequence of the four different genes of beta defensin. The phylogeny was constructed by the program Mega 4.

Sequencing and Sequence Analysis of the Amplified Defense Genes: One band from each gene was excised from the gel, purified and sequenced. The sequence analysis revealed that all the obtained DNA nucleotide sequences belong to human defense genes. The phylogenic tree was constructed based on the obtained DNA nucleotide sequence revealed that (Fig. 7); there is a relationship between the defensin gene beta HBD1 and the HBD3. Whenever, the defensin gene HBD2 was closely related to HBD4. Moreover, the four examined genes came from one ancestor which indicates that the system of defense in the human saliva have a unique behavior for defense against the pathogenic bacteria.

DISCUSSION

Oral cavity is a reservoir of bacteria with high species diversity, not all species can cause invasive disease in patients. Identification of bacterial pathogens in oral lesions is challenging because cultivation of specimens from this non sterile site invariably yields a diversity of bacteria, making associations between cultivated bacteria and particular oral lesions tenuous [21]. Furthermore, histological detection of bacteria in tissues by means of Gram staining can be difficult, because some Gram-negative bacteria are not easily distinguished among the background of tissue cells [22]. Molecular methods such as PCR and fluorescence in situ hybridization (FISH) can be used to detect, identify and localize bacteria associated with occult bacterial lesions of the mouth [22].

In this study saliva samples were collected from school children randomly to examine the bacterial biodiversity especially these students were of many nationalities. Biological and molecular methods revealed that only four different species were obtained (aerobic only). According to Daivd *et al*. [23], Gram stain and morphological characterization are not enough to identify the bacteria. Moreover, the 100 bacterial isolates were reduced into 30 isolates based on the colony size, colony morphology, Gram stain and microscopic examination.

The 30 bacterial isolates should be categorize and fingerprinted using the molecular tools to reduce their number before performing the 16S r RNA sequencing for them. RAPD-PCR was performed for the 30 bacterial isolates using different 5 arbitrary primers and the results revealed that five different categories were observed. Dal Bello and Hertel [24] used RAPD-PCR for genotyping of lactobacilli in human saliva and they reported that RAPD-PCR analysis indicated that several strains of these species are present both in the oral cavity and in the fecal samples of the same subject. Oral isolates of the species *L. gasseri and L. vaginalis* showing

identical RAPD types were found to persist over time, suggesting that these species are autochthonous to the oral cavity.

For identification of the five different groups obtained by the RAPD-PCR, only one representative bacterium was selected randomly and their genomic DNA was subjected to 16S rRNA gene amplification using specific PCR. The sequence analysis revealed that the bacterial isolates are Streptococcus mutans, Porphyromonas gingivalis, Staphylococcus epidermidis, Streptococcus salivarius and Lactobacillus sp. The isolates were similar with the other related bacteria in Gene Bank with percentage ranged from 99 to 100. Similar results were obtained by Jung-Gyu *et al*. [25] who examined the bacterial biodiversity in the human mouth among the Korean peoples with different ages. Also they reported that there have been several reports in Korea on the bacterial species from patients with oral diseases [26-27], but there has been no comparative report from periodontal healthy visitors.

For studying the human response against the mouth invested bacteria, saliva of some persons containing five different bacterial species were examined using four genes belong to beta defensin family. The oral epithelium functions as a mechanical and protective barrier to resist bacterial infection [28]. Human beta-defensin-2 and -3 (hBD-2, -3) are small inducible antimicrobial peptides involved in host defense. Antimicrobial peptides are important contributors to maintaining the balance between health and disease in this complex environment. These include several salivary antimicrobial peptides such as β- defensins expressed in the epithelium and LL-37 expressed in both epithelium and neutrophils. Results revealed that different amplicons with different molecular sizes were obtained. We suggested that the different amplicons obtained with one set of primers of specific genes, may be different copies of the same genes. The recent theory reported that the high copy numbers of the defense genes the high strong immune system including them [22- 26]. Feucht *et al*. [29] studied the induction of human beta-defensin mRNAs by *Actinobacillus actinomycetemcomitans* in primary and immortalized oral epithelial cells and they reported that the increase in hBD-3 gene expression is dependent upon viable bacteria and not their lipopolysaccharides (LPS). Inflammation and destruction of periodontal tissues are considered to result from the response of a susceptible host to a microbial biofilm containing Gram negative pathogens. Meyer *et al.* [30- 31] reported that *Candida albicans (CA)* in oral cavity induce the Beta defensin gene and revealed that *hBD-2* shows a potent antimicrobial activity against CA. It can be concluded that beta defensin genes are presented as a defense system against the pathogenic bacterium and they work in preventing the epithelial inflammation.

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