MANSOURA JOURNAL OF BIOLOGY
Official Journal of Faculty of Science, Mansoura University, Egypt
E-mail: scimag@mans.edu.eg ISSN: 2974-492X

Comparison and Differentiation of Oral Microbial Flora from Healthy and Diabetic Individuals

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Received:22/9/2019
Accepted:28/9/2019

Abstract: Oral microbial flora constitutes an important part of the total human microbiome and any imbalance of this community is directly reflected upon the oral and whole-body health and disease status. Diabetes mellitus affects the mouth environment in terms of the flow of saliva and hence nutrients availability for mouth microbes. Studying and comparing the mouth cavity microbial flora of healthy and diabetes represented a mystery needed to be discovered. The use of the available molecular techniques such as protein and DNA fingerprinting were adopted to answer and expanded our knowledge about this part of the human body environment. The molecular methods were not only superior to phenotypic methods of bacterial identification but they were faster and conclusive. It is highly important to note the ability of the molecular techniques to distinguish between strains of the same species; either at the protein or DNA levels. Moreover, it is an obligation to state these methods works well with cultivable bacteria and other methods depending on DNA sequencing and 16S rRNA would be important for non-cultivable microbes **.**

keywords: Oral, microbiomes, diseases, molecular and phenotypic**.**

1.Introduction

The oral flora comprises a diverse array of organisms which include bacteria, fungi, mycoplasma, protozoa and possibly viruses. Bacteria by far are the most predominant group of organisms exists in the mouth cavity of normal healthy people. Only 40-60% of the oral cavity bacteria remain uncultivable under the existing laboratory culture methods [1]. Variability of the oral microflora in normal healthy individuals and others suffering from different kinds of illnesses is a challenge. A large portion of world population suffers from diabetic mellitus (DM); which is basically is a metabolic disorder. Patients suffer from either type of this disease (type 1 or II) show signs of mouth dryness and this must affect the microbiota of the mouth cavity. However, the richness of the mouth cavity is evident from the continuous supply of nutrients, high humidity and variable oxygen concentrations, the existence of soft tissues like gingiva and hard non-shedding supporting tissues such as teeth support the flourish, establishment, and onset of microbes and subsequent interaction with various host cells. This is reflected on the contribution of the mouth cavity microbial population on worsening the overall health status of diabetic patients [2].

The increasing incidence of DM cases around the globe has alerted the World Health Organization (WHO) to be concerned and revealed that about 171 million people have DM worldwide and expected it will rise to more than 366 million by 2030. Whereas, in 2016, the International Diabetes Federation reported the number of DM cases to be about 387 million and estimated it will reach 592 million by 2035. Many other studies have predicted even higher numbers and prevalence of caries among DM patients with poorly controlled DM. [3, 4]. This sizable number of cases and the complications they suffer have driven scientists, especially microbiologists, to examine any correlation between the mouth microbial population and severity of DM [5].

Due to the poor oral hygiene and richness of the microbial community (aerobic and anaerobic) in the oral cavity diabetic patients suffer from complicated oral infections and

tooth loss. To avert the seriousness of the infection, diabetic patients not only misuse but abuse the available antibiotics in the treatment of their infections which contributes to enhancement and development of infectious agent resistance to antibiotics[6]. The irrational use of antimicrobial drugs has led to the emergence of Multi-Drug resistance (MDR) among the various strains of microorganisms inhabiting mouth cavity [7-9].

Commensal bacteria form biofilms in healthy people and provide benefits to the host. Within these biofilms, oral disease is correlated with a change in bacteria equilibrium to pathogenic species. Cells growing in biofilm often display modified phenotypes such as enhanced antibiotic resistance (10-1000 fold) (impeded drug penetration of EM) and reduce immune system clearance . These biofilm-specific phenotypes confer enhanced disease-causing potential on pathogens, and estimated 60 % of all bacterial diseases caused by bacterial biofilm, according to the National Institute of Health (NIH)and USA [10] and [11].

Great advancements in molecular biology and modern spectroscopy technologies such as matrix assisted laser desorption ionization (MALDI) have been applied in the field of microbial identification and differentiation beside PCR and RAPD-PCR fingerprinting.

Material and methods

1. Sample collection

Samples (swabs) were taken from normal and diabetic indivduals randomly; 20 samples from each group. Each swab was individually rapped and transferred immediatetly to the laboratory for bacterial isolation.

2. Bacterial isolation and growth media

Each individual swab was streaked onto the surface of LB agar plate (LB medium contains g/L: peptone 10, yeat extract 5, and sodium chloride 10 and solidified by 15g agar-agar) and incubated at 37°C for overnight. LB broth cultures were kept in a shaking incubator (180 rpm) at 37°C for overnight (Cappuccino and Sherman2001). The isolated colonies were purified, selected and stored at -80°C in 20% glycerol stocks for longer preservation time. The bacterial colonies were morphologically, biochemically and molecularly characterized.

3. Identification of Bacteria

Standard bacteriological techniques used to identify all bacterial isolates included Gram's stain [12-15] growth on specialized media to test for multiple characters such productin of catalase [16], production of DNase [17], growth on congo red agar [18]., hemolysis of blood [15], fermentation of mannitol sugar [19], and type of growth on Baird-Parker agar plates [20].

4. Bacterial proteins by electrophoresis in denatured polyacrylamide gel:

Sodium dedocylsulfate polyacrylamide gel electrophorosis (SDS-PAGE) described by [21]. was used for protein banding patterns of all bacterial isolates obtained in this study. It is the standard method for separating and differentiating total cellular proteins according to their molecular weights and hence it is used to build a protein fingerprint for each isolate.

5. Random amplified polymorphic DNA analysis (RAPD-PCR)

Bacteria inoculated in LB agar at 37^{o}C for overnight and their genomic DNAs were isolated according to instructions of the protocol supplied by the manufacturer of the kit used (Thermo Scientific, Germany). The purified genomic DNAs were used as template for the RAPAD-PCR reactions with four random primers selected from the Operon kits (OPU-16 5′-CTGCGCTGGA-3′), (OPT-16 5′-GGTGAACGCT-3′), (OPK-02 5′-GTCTCCGCAA-3′) and (OPI-17 5′-GGTGGTGATG-3′). Each of the reaction mixtures was adjusted to a total volume of 20µl containing 1µl DNA template, 4µl 5xbuffer, 2µl primer, 0.5µlTaq DNA polymerase (1 enzyme unit) and 12.5µl dist. Water. The RAPD-PCRs started by denaturing the template DNA at 94^{o}C for 3 minute followed by 40 amplification cycles. Each cycle consisted of denaturation at 94^{o}C for 1 min, annealing the primers with the denatured DNA template at 30^{o}C for 30s, the nascent product was extended at 72^{o}C for 1 min, at the end of the 40 cycles an extension step at 72^{o}C for 5 min. was performed The products of RAPD-PCRs were electrophoresed in agarose gel in the presence of ethidium bromide and photographed. [22]

Results

Forty bacteria were isolated from the collected 40 swabs from healthy and diabetic individuals. The initial screening revealed that six isolates from healthy individuals (8h, 9h, 13h, 15h, 20h and five from diabetic patients (D2, D 3, D6, D14, D16, and D19) appeared to be yeast-like microbes. The did show budding upon staining with Gram's stain. The remaining 29 isolates looked like typical bacteria. All bacterial isolates were further identified according to Bergey's Manual beside some molecular biology techniques.

All of the 29 bacterial smears from healthy (15 isolates) and diabetic people (14 isolates) were purple in color (positive) when stained with Gram's stain. Ten of the 15 bacterial isolates from the healthy individuals looked cocci (1h, 2h, 4h, 5h, 7h, 14h, 22h, 23h, 24h, 25h), four rod-shaped (3h, 6h, 18h and 27h) and only one isolate (10h) looked as coccobacillus and stained negative to Gram stain. Thirteen out of the 14 bacterial isolates from diabetics were Gram-positive and only the rod-shaped (D4) isolate showed negative response to Gram's stain. Five from the 13 remaining isolates were cocci (D5, D7, D8, D18, D20) and eight were bacilli (D1, D9, D10, D11, D12, D13, D15, D17).

Nine bacterial isolates showed resistance to number of the tested antibiotic discs. These isolates were further identified by biochemical and molecular methods.

1. Biochemical Identification of selected bacterial isolates

Five of the multidrug resistant isolates (MDR) were isolated from healthy individuals (4h, 10h, 14h, 23h, 25h) and four (D4, D8, D9, and D15) were from diabetic patients. These nine isolates were cultivated on selective media and subjected to several biochemical assays and growth on selective media for identification as stated above in the materials and methods section.

1.1 Cultivation on Baird-Parker Medium

All purified bacterial isolates from the mouth swabs were first streaked onto Baird-Parker medium. Only one the streaks turned black surrounded by a halo, characteristic of *S. aureus* growth on this medium (Fig. 1). This type of growth on this Baird-Parker medium considered a presumptive identification of standard *S. aureus,* which needs more verification.

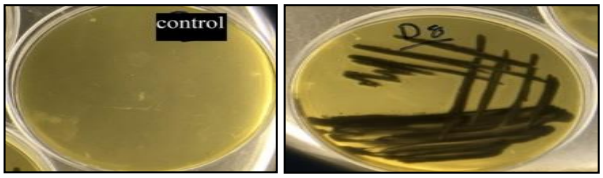

Fig. (1): Cultivation of on selective media show black colonies.

2.2 Blood hemolysis

All MDR isolates from either health or diabetic individuals were non-hemolytic except isolate D8 which showed β-hemolytic activity (Fig. 2). Growth on this enrichment medium is helpful in determining of any of the nine isolates belongs to either staphylococci or streptococci.

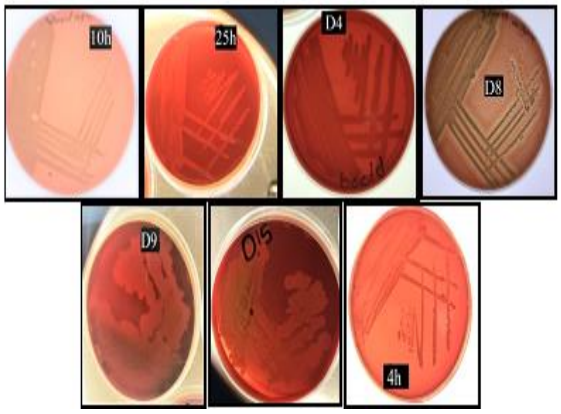

Fig. (2): Blood hemolysis test of the seven MDR local uisolates from oral cavities of some healthy and diabetic individuals.

2.3 Fermentation of mannitol sugar

Only isolate D8 was able to change the indicator color (phenol red) from red to yellow (Fig. 3). Growth on mannitol-salt agar is both selective and differential medium in terms of high salt tolerance (7.5% sodium chloride) and ability to produce acid due to fermentation ion of mannitol sugar. identification and enumeration of the *Staphylococcus aureus* from any source.

Fig.(3): Growth of selected bacterial isolates on mannitol-salt agar isolates (4h, 25h and D8).

2.4 Catalase Test

Distinction between aerobic and anaerobic bacteria temporally residing in the mouth cavity of healthy and diabetic was done using catalase test. These bacteria included staphylococci, streptococci, Entercoccous, Clostridium, and Bacillus species. The results of the catalase test indicated that isolates D8, 4h, 14h, 23h and 25h were catalase positive.

2.5 DNase Test

This differential test depends on two major characters: the fading of the green color of the included indicator compound (methyl green) and the appearance of a clear halo around the DNAase positive test organism. None of the isolated bacteria were grown on DNA containing agar plates in the presence of a color indicator, these were 4h, 10h, 14h, 23h, 25h, D4, D8, D9 and D15 (Fig. 4). Only isolate D8 was able to use DNA as a carbon source for growth; degraded it and surrounded itself with a clear halo and the green color was faded.

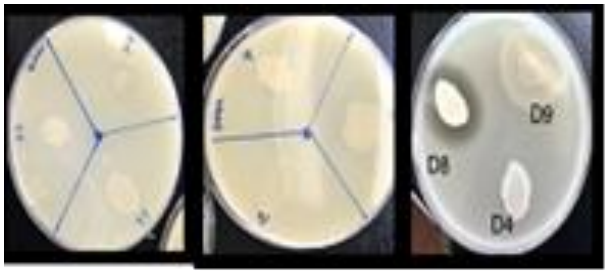

Fig. (4): DNase activity test for eight bacterial isolates were negative (4h, 10h, 14h, 23h, 25h,D4,D8,D9 and D15) and the D8 was the only positive DNase producing isolate.

2.6 Biofilm production test

A biofilm is a major virulence factors that contributes to pathogenicity. The MDR bacteria protect themself from the host's immune system and administered antibiotics by forming biofilms. Distinguishing biofilm-forming bacteria from none biofilm-forming ones was possible by growing the isolated bacteria from healthy and diabetic patients on Congo-red agar plates (CRA) (Fig. 5).

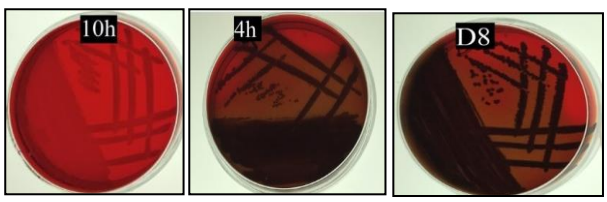

Fig. (5): Growth of biofilm positive vs control bacterial isolates on CRA plates.

3. Molecular characterization:

Various molecular biology techniques were used to identify bacterial isolates from healthy and diabetics at the species level. Some of the techniques used in this study were plasmid profiling, protein banding patterns, DNA fingerprinting using randomly amplified polymorphic DNA (RAPD-PCR).

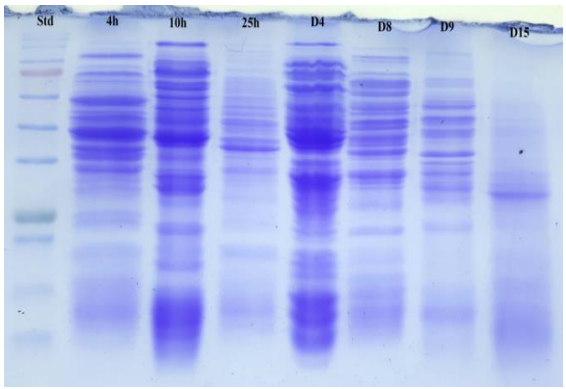

Fig. (6): Total cellular proteins of seven MDR isolates. All lanes are marked with the initial isolate numbers and lane Std: protein marker in ascending order; in kDa.

Quick examination of the protein banding patterns of the seven MDR seven isolates showed that all are different from each other. The protein patterns of isolates 10h and D4 look awfully close but closer examination showed the difference in the electrophoretic motilities of band with apparent molecular sizes 245, 180, 135, 100, 75 ,63 ,48 ,35 ,25 ,20 ,17 ,11 , top to bottom kDa. The distinction is clear at the high or low molecular weight proteins.

3.2 Random DNA fingerprint of the MDR local isolates

The DNA fingerprinting of three isolates from healthy people (4h, 10h and 25h) using four random primers showed distinctive differences (Fig. 7). Primer OPU-16 produced four DNA bands with the 4h isolate (1123bp, 747bp, 527bp and 331bp), two bands with isolate 10h (925bp slightly faint, 542bp sharpest), and one DNA fragment with isolate 25h (423bp). Primer OPT-16 produced three DNA fragments with isolate 4h (1123bp, 890bp and 247bp), four bands with isolate 10h (1401bp faint, 872bp Sharpest, 730bP faint, 337bp sharp), and four bands with isolate 25h (620bp, 499bp, 273bp and 208bp). Primer OPK-02 was not reactive with all isolates and produced no DNA bands, and primer OPI-17

amplified single DNA fragment with isolate 4h (999bp), two bands with isolate 10h (403bp sharpest, 318bp faint), three DNA bands with isolate 25h (119bp, 472bp and 379bp).

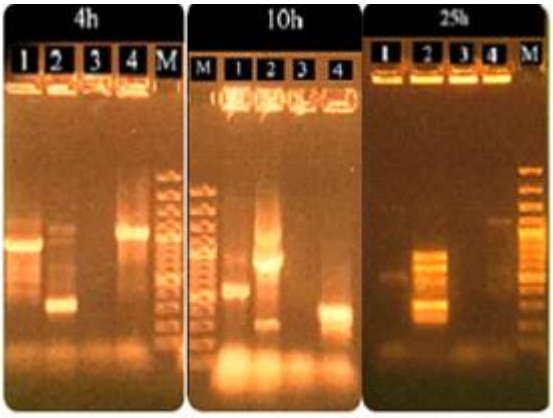

Fig. (7). RAPD-PCR fingerprints of three local isolates (4h, 25h and D8) using four Operon random primers OPU-16 (lane 1), OPT-16 (lane2), OPK-02 (lane 3), and OPI-17 (lane 4). Lane M contains DNA molecular weight marker (3,000 to 100bp).

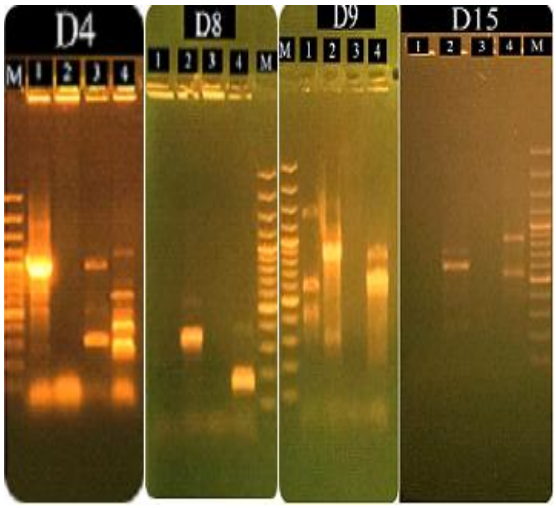

Fig (8): RAPD-PCR fingerprints of four local isolates (D4, D8, D9 and D15) using four Operon random primers OPU-16 (lane 1), OPT-16 (lane2), OPK-02 (lane 3), and OPI-17 (lane 4). Lane M contains DNA molecular weight marker (3,000 to 100bp).

The DNA fingerprinting of four isolates from diabetics people (D4, D8, D9 and D15) using four random primers showed distinctive differences (Fig. 8). Primer OPU-16 produced three DNA bands with the D4 isolate (864bp, 690bp and 491bp), no DNA bands with isolate D8 , and three DNA fragment with isolate D9 (156bp, 596bp and 432bp), no DNA bands with isolate D15. Primer OPT-16 no DNA bands with isolate D4, two bands with isolate D8 (297bp and 507bp), and two bands with isolate D9 (313bp and 965bp), two bands with isolates D15 (432bp and 535bp). Primer OPK-02 produced four DNA bands with the D4 isolates(969bp, 645bp, 459bp and 267bp), and no DNA fragment for (D8, D9 and D15) isolates. Primer OPI-17 produced six DNA bands with the D4 isolate (1271bp, 690bp, 602bp, 429bp, 350bp and 233bp), two DNA bands with isolate D8 (150bp and 330bp), three bands with isolate D9 (550bp, 660bp and 867bp), two bands with isolates D15 (520bp and 663bp).

Discussion

Microbial number in human body highlighted their importance to human health in general and microbes residing in the oral cavity are no exception. Accurate and rapid identification of these microbes such as bacterial is of high values to determine their validity to human. Identification based on phenotypic characterization such as growth on specific media (blood agar. biofilm production, Congo red, fermentation of mannitol sugar, or growth on Baird-Parker or enzymatic activity such as DNase and/or catalase activity) suffers from several exhaustively said limitations in terms of needed expenses, time, and labor. Besides the inconsistency in the identification which may be attributed to known strain variation within each bacterial species. Currently, new methods based on molecular fingerprinting replaced the phenotypic ones and avoided their shortcomings. These included protein banding by electrophoresis and RAPD-PCR typing accurately characterized and identified the minute differences among the different isolates during this study. Molecular methods did contribute to discovering the great diversity of the oral microbiome which added to the knowledge of their role in health and disease of such environment. This diversity was accompanied by genetic heterogeneity, as well. Moreover, no distinction was detected in the bacterial population inhabiting the oral cavities of healthy people and diabetics residing in Mansoura City, Egypt. A striking similarity was evident in the protein banding patterns of 10h bacterium isolated from healthy individuals and D4 bacterium isolated from the diabetic patient, respectively. Our results identified the different bacteria isolated to be [23-31].

The fact that oral cavity structures are many and each part attracts specific types of the bacterial population. These bacteria may be involved causing specific oral diseases such as caries, and periodontitis or other human diseases such as endocarditis, osteomyelitis, and cardiovascular. Therefore, the study of the mouth bacterial flora among healthy and diabetics are of great importance, since diabetics are more prone to dental diseases than healthy people.

Therefore, we expect variability and diversity among the local flora of mouth and our data supported this hypothesis where the isolated obtained were limited to few bacterial genera of the more common inhabitants of the oral cavity[32].

had reported that more than 700 species constitute the oral cavity bacterial microflora despite the inclusion of all mouth cavity parts. They used molecular techniques such as 16S rRNA amplification of the oral metagenomic not cultural techniques in their study and found that almost 50% of the detected bacteria cannot be cultivated on the known bacteriological media. This supports the limited number of bacteria isolated in our study. They also noted the diversity among the different bacterial species residing in different mouthparts as well as the specificity. More importantly, they recommended a comparison between the microflora of healthy individuals before linking these resident microbes to oral diseases. Other studies on oral microflora had been reported by … and had reported over 500 species not only in healthy people but in HIV patients with periodontal diseases. [33, 34]. had reported significant diversity and differences between healthy and normal people suffering from subgingival and supragingival plaques.

Generally, the molecular methods need specific experiences and a laboratory setup which may not available all the time. Among these techniques dependent on using PCR as in our case RAPD-PCR are the best to identify even the least amount of differences or a single nucleotide polymorphism in a single copy of the DNA from clinical samples[35, 36]. Other investigators have used similar techniques to determine the bacterial diversity of saliva, subgingival plaque of a subject with gingivitis, and dent alveolar abscesses [37-40]

The limitation in this study is the dependence upon the only cultivable bacteria but the term microbiome includes also the non-cultivable ones. The last group of microbes cannot be studied by either the traditional or molecular techniques used during this study' as long as these bacteria cannot be cultured. However, we suggest the use of culture-independent more advanced molecular techniques such as 16S rRNA-based microarrays, and the highly sophisticated next-generation DNA sequencing (NGS) including pyrosequencing, real-time single-molecule DNA sequencing, and nanopore-based sequencing.

In conclusion, the recent advancements in molecular biology techniques had facilitated early diagnosis of bacterial pathogens causing periodontitis, dental caries, and other mouth cavity diseases. These molecular methods also have broadened our experiences and knowledge about the important roles played in our health and disease by oral flora. We also suggest heavy dependence on these techniques for the discovery of all wonders of the mouth cavity microbiome.

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