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Research Article

Phenotypic and Genotypic Characterizations of Non-Biofilm Producer *Porphyromonas gingivalis* Associated with Periodontitis in Al-Najaf Al-Ashraf/Iraq

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ABSTRACT

This study aims to investigate the correlation between some virulence factors and biofilm production among *Porphyromonas gingivalis* associated with periodontitis. One hundred and two sub gingival swabs have been collected from patients suffering from acute and chronic periodontitis admitted to Specialty Dental center in the Holy city of Najaf and specialized clinics in the Faculty of Dentistry of the University of Kufa from both sexes during the period from September 2017 to January 2018. A total of 34 isolates (28.43%) were identified as *P. gingivalis* by amplification of *16S rRNA* using PCR technique. *luxS*, *lux pro* and *lux down* genes were investigated and the results of PCR showed that none of the strains were possess *luxS*, 3 strains of *P. gingivalis* were possess *lux pro* and 2 strains of *P. gingivalis* were possess *lux dwn*. Only 10 strains of *P. gingivalis* were selected for detection of some virulence factors. The results showed that all strains were unable to produce biofilm with variant abilities of bacterial strains for autoaggregation have been observed (decrease in O.D of bacterial growth after 1 hr. of incubation), and one strain gave +ve results for hemagglutination.

Keywords: *P. gingivalis*, biofilm, auto-aggregation, PCR.

INTRODUCTION

Porphyromonas gingivalis is obligate anaerobic Gram negative bacteria which is represented the main cause of periodontitis, dental cysts and endodontic infection in humans[1]. It's also a keystone in the development and progression of periodontal disease[2] for its ability to destroy host defense mechanism[3,4]. It also plays an important role in the transformation of gingivitis from benign tooth decay into pathogenic microbial communities[5]. The susceptibility of *P. gingivalis* for colonization and reproduction in the oral cavity is affected by its ability to participate in the oral biofilm[6]. *P. gingivalis* is characterized by possessing many factors of virility that enable it to cause infection in the body of the host. These include: (i) Fimbriae and hemagglutinin: colonization agent used by bacteria to invade host tissue. (ii) Gingipain: which destroys host cells and proteins. (iii) Lipopolysaccharide: it's an internal toxin. (iv) Outer membrane vesicle: which contain lipopolysaccharide and gingipains. (iiv) Capsule: which increases bacterial resistance to phagocytosis and serum factors[7,8].

The tooth associated with biofilm plays an important role in the onset and progression of periodontitis. The biofilm forms a protective barrier for bacterial cells from the host's immune system and makes them resistant to antibiotics[9,10]. The formation of periodontal pathic biofilm includes three essential stages. The initial stage of early colonization are mediated by two types of oral microorganisms, *Actinomyces* and *Streptococcus*. The formation of biofilm starts with their adhesions to the lining of the oral cavity by different types of adhesion factors such as fimbriae and polysaccharide. This stage is followed up by colonization of oral cavity by species of microorganisms which are considered intermediate colonizers such as *Fusobacterium nucleatum*. They strengthen the assembly associated with late colonizer such as *P. gingivalis* which is the main causative of gingivitis (the final stage of biofilm formation) helping in the growth of an entire microbial community in the biofilm[11].

The mouth is the main gate of digestive system and the rest of the body depends mainly on mouth and teeth. For the importance of teeth, the spread of periodontal disease, and lack of cultural and health awareness in this area the present study was intended to investigate the role of *P. gingivalis* in periodontal disease and the importance of the biofilm produced by it in the pathogenicity of this bacteria.

MATERIALS AND METHODS

Samples collection

A total of 102 samples (46 samples were taken from males and 56 samples were taken from females) were collected by using a special brush from patients whom admitted the Specialized Center of Dentistry in the holy city of Najaf and Teaching Hospital / College of

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Table 1: The oligo-synthesis nucleotide sequences (Alpha – Canada).

Genes	Oligonucleotide Sequenc ′5 ⟶ ′ 3		Size of Amplicons	References
16SrRNA	F	AGAGTTTGATCCTGGCTCAG	500bp	
	R	TTACCGCGGCTGCTGGCAC		14
luxS	F	CCGTCGCTACATCGAGTACC	409bp	
	R	CGAGGCATATATGTCTCCCG		
Lux pro	F	GAGGATCTTCTCGCCCTTTT	438bp	
	R	CGAGGCATATATGTCTCCCG		15
Lux dwn	F	GTGCCGTCTGATTCACATT	229bp	
	R	CCGTCGCTACATCGAGTACC		

Dentistry / Kufa University with acute and chronic periodontitis and gingivitis of different ages and both sex during the period from September 2017 to January 2018. All samples were cultured by streaking method on both Blood Agar Base and Brain Heart Infusion Agar supplemented with 5% sheep blood, 1µg/ml of vitamin k, 5µg/ml of hemin and incubated und eranaerobic condition for (7-14) days for initial isolation of bacteria.

Identification of P. gingivalis

The bacterial isolates were identified by conventional test depending on morphological characters of colonies (color, size, texture and shape of edges) and microscopic test[12,13]. Black colonies were further identified genetically using *16S rRNA*.

Molecular identification

PCR technique was used to confirm the identification of *P. gingivalis* isolates and detect some virulence genes associated with biofilm production such as *luxS, lux pro,* and *lux dwn.*

DNA Extraction

Extraction of bacterial DNA using Boiling method has been follow as described previously (26). Briefly bacterial cell suspension boiled for 5 minutes then incubated in water bath at boiling temperature for 5 minutes, then, ice bath incubation for another 5 minutes. The lyses mixture was centrifuge at 15000 rpm/min then, the DNA was precipitate by mixing with isopropanol for 24 hrs and centrifuged again at 10000 rpm/min. The DNA precipitate was conserve in TE solution and DNA concentration was measure by DNA-RNA spectrophotometer (Bio-Droop).

Amplification process

The oligo-synthesis nucleotide sequences (Alpha - Canada) shown in table (1) were prepared with a final concentration of 10 pmol/µl. PCR mixture was prepared with a final volume of 20 µl consisting Maxime PCR Premix(iTaq, iNtRON - Korea), 5µl for both forward and reverse primers, 6µl of DNA template and 4µl of nuclease free water. The amplification conditions of the thermocycler system (Biometra - Germany) were determined as shown in table 2.

The amplification products were electrophoresed on agarose gel (1%) stained with Ethidium Bromide at 100 volt for 50 minutes and. Gel documentation system (Optima - Italy) was used for visualizing and photographing the gel.

Phenotypic Detection of Biofilm Production

The method described by[16] was used to detect the ability of *P.* gingivalis for biofilm formation by using tissue culture plate. Initially the tubes containing bacterial growth were centrifuged at 5000 cycle/ min for 10 minutes. The cells were washed twice with PBS (Himedia - India) and then re-suspended in 1ml of PBS and TSB 1:2. An amount of 150µl of bacterial suspension was added to each well (3 replicates to each isolate were used) and then incubated anaerobically for 24 hours. The plates were washed twice by distilled water after the culture supernatant was removed and left to dry for 1h., then stained with 0.5% safranin and left for 15 min. The wells were washed twice with distilled water and 95% ethanol was added to solubilize the safranin for 5 min and the optical density was measured at 490 nm.

Detection the ability of bacterial autoaggregation

The method of [17] was used. Briefly, tubes containing bacterial growth were centrifuged at 5000 rpm/min for 10 min. after incubation for 48 hours. The bacteria were re-suspended by PBS (Himedia - India) after washing twice with the same buffer. The turbidity of the suspension were adjusted to O. D_{660} 1.0 by using spectrophotometer (Tuiup international corp - Italy). Five ml of each sample were transported to a new tube and incubated in shaker incubator at 37°C for 120 rpm/min. the turbidity was measured after 60 min. The decrease in turbidity refers to autoaggregation.

RESULTS AND DISCUSSION

The result of isolation and identification of *P. gingivalis* based on phenotypic characters and microscopic examination showed that 39 bacterial isolates belong to *P. gingivalis*, while the result of PCR for amplification of *16S rRNA* showed that 34 isolates belong to *P. gingivalis* by showing amplicons with molecular weight 500 bp (Figure 1).

Molecular Detection of Biofilm Coding Genes

The results of agarose gel electrophoresis of amplicons obtained from the amplification of *luxS* showed that no isolates were carried this gene. On the other hand, the results of agarose gel electrophoresis of amplicons obtained from the amplification of *lux pro* and *lux dwn* showed that 3 isolates of *P. gingivalis* were carried *lux pro* gene by appearing of amplicons with molecular weight 438bp. Only 2 isolates of *P. gingivalis* were carried out *lux dwn* by appearing of amplicons with molecular weight 229 bp (Figure 2)

Biofilm Production Test

The results showed that all bacterial isolates were unable to produce biofilm. Biofilm is one of the important virulence factors possessed by most bacterial species.

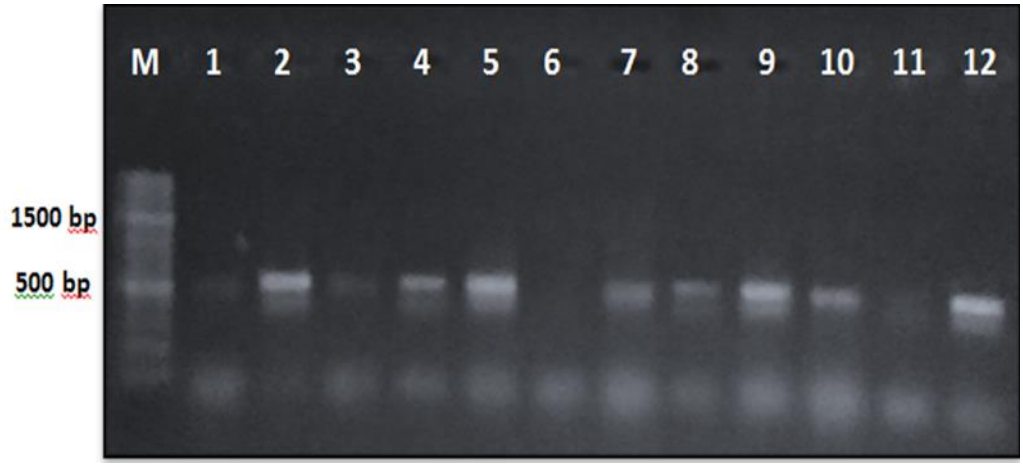

Figure 1: Agaros gel electrophoresis of *16S rRNA* amplicons of *P. gingivalis* (500bp). (1% agarose at 100 volt for 50 min) Lane M: DNA marker (100bp); Lane1-12 except Lane 6 positive results for amplification.

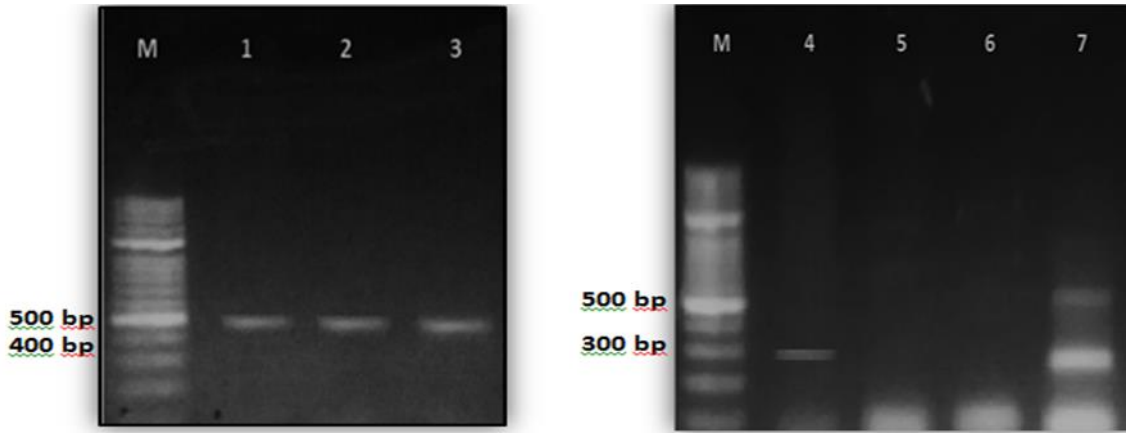

Figure 2: Agaros gel electrophoresis of *lux pro* and *lux dwn* amplicons of *P. gingivalis* (1% agarose at 100 volt for 50 min) Lane M: DNA marker (100bp); Lane1,2,3 positive results for amplification of *lux pro* gene (438 bp). Lane 4,7 positive results for amplification of *lux dwn* gene (229 bp).

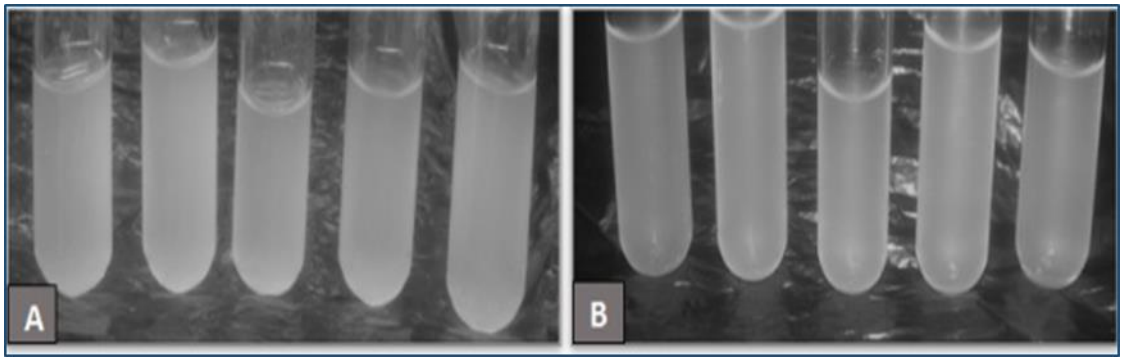

Figure 3: The ability of *P. gingivalis* for autoaggregation. A- Test tube prior to incubation in the shaker incubator. B- Test tube after an hour of incubation in the shaker incubator.

Several studies have indicated the susceptibility of *P. gingivalis* to the production of biofilm[18]. manifested that all isolates of *P. gingivalis* have the ability to produce biofilm and that half of the isolates have a high ability to produce biofilm while the other half have a moderate ability to produce biofilm. The susceptibility of *P. gingivalis* to produce biofilm is influenced by a combination of factors such as gingipains, hemagglutinin, fimbriae and capsule[19-21]. Several studies have indicated that there is a variability in the ability of *P. gingivalis* strains to form biofilm, whereas *P. gingivalis* 381 has a great ability to form biofilm. *P. gingivalis* W83 is not able or has a weak ability to form biofilm[22-24]. *P. gingivalis* isolates without *lux pro* and *lux dwn* genes incapable of producing biofilm due to the presence of capsule that plays role in the inhibition of biofilm formation because of the loss of its adhesion ability. *P. gingivalis* W83 strain could also produce capsule causing spread infection in different organs in infected mice such as blood, spleen, and kidney of mice[25,26] compared with

Table 2: Amplification conditions of polymerase chain reaction.

Genes	Initial Denaturation (ºC/min)	No. of Cycles	Condition of Cycle			Final extrnsion (ºC/min)
			Denaturaton (ºC/sec)	Annealing (ºC/sec)	Extension (ºC/sec)	
luxS	94/2			47/30	72/60	72/5
Lux pro	94/2	35	94/30	53/30	72/60	72/5
Lux dwn	94/2				72/60	72/5

non-encapsulated strains which lead to localized infection because of its adhesion ability[27,28,16]. The reason could also be its inability to produce biofilm due to the inhibitory effect of some chemical compounds produced by *Streptococcus or Staphylococcus* that accompany *P. gingivalis* bacteria in periodontal infection caused by *P. gingivalis*[29]. The production of arginin deaminase from *Streptococcus* bacteria accompanying *P. gingivalis* W83 inhibition leading to suppressing monospecies biofilm formation by *P. gingivalis* 381[30].

Autoaggregation Ability test

The measurement of optical density of bacterial isolates grown on Brain Heart Infusion broth for 48h showed that only 5 isolates had an optical density of 0.99 – 1.0. These isolates were selected to detect the ability of these isolates for autoaggregation. The results showed a decrease in the value of the optical density after an hour of an incubation indicating the beginning of the ability of bacteria for autoaggregation (Figure 3). The results also showed a difference in the values of the decrease in optical density ranged between (0.7 – 0.9).

The process of autoaggregation of bacterial cell is one of the necessary processes for the beginning of the formation of the biofilm[31,32]. Both large and small fimbriae play an important role in biofilm formation. Some studies have suggested that the presence of large fimbriae enhances autoaggregation while small fimbriae inhibit it[33-35)]. Also[31] mentions that small fimbriae are essential for the autoaggregation playing an important role in the formation of biofilm.

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