GENOTYPIC AND PHENOTYPIC DETECTION OF SOME VIRULENCE FACTORS AMONG *Porphyromonas gingivalis* RELATED WITH PERIODONTITIS IN AL-NAJAF AL-ASHRAF CITY, IRAQ

Alaa Shahid Jassim AL-Bdery and Ahlam Kadhum Al-Yasseen*

Department of Biology, Faculty of Education for Girls, University of Kufa, Iraq.

Abstract

*Porphyromonas gingivalis* represented one of the most etiological agents in pathogenesis and progression of the inflammatory events of periodontal disease. Fimbriae and gingipains are the most potent factors responsible for the attachment of bacteria to the host, damage the periodontal tissues, inactivate and degrade a number of host defense and structural proteins, and persistent infection of *P. gingivalis*. This research aimed to explain the distribution of *P. gingivalis* among oral cavity in patient suffering from periodontitis and to detect the correlation between biofilm formation and adherence with Fimbriae and gingipin encoding genes. Isolation and identification of *P. gingivalis* from patients suffering from chronic periodontitis (CP) according to phenotypic detectable of biofilm formation and the capacity of bacterial cells to adherance to oral human epithelial cell has been carried out. Out of 150 subgingival dental plaque specimens only 78 isolates were belong to *P. gingivalis* by conventional methods while the results of PCR technique showed that only 20 isolates were belongto *P. gingivalis*. Genotypic detection of *fimA* genotypes revealed that *fimA* type IV gene was the most predominance (20%) followed by *fimA* type III while *fimA* type II and V show up the lowest percentage (5%) whereas *fimA* type Ib was not identified. Genetic investigation of *kgp* and *rpgA* revealed that all isolates were possess both genes. All isolates have the ability to produce biofilm and adherence to oral human epithelial cells. Wide distribution of *P. gingivalis* in CP patients and molecular methods were more accuracy in identification of these bacteria. A wide prevalence of *fimA* genotypes ( except *fimA* Ib), *kgp* and *rpgA*. A correlation has been found between biofilm formation, adherence to epithelial cells and possessing of *fimA* genotypes.

Key words : *P. gingivalis*, *fimA* genotypes, periodontitis, PCR.

Introduction

*Porphyromonas gingivalis* is an opportunistic pathogen associated with up to 85% of periodontitis and its existence at the infection site is indicate of disease progression (Califano et al., 2003). It is an anaerobic Gram negative bacteria, non-motile, asaccharolytic, short rods or coccobacilli, non-sporeforming and forms black-pigmented colonies on blood agar plates (How et al., 2016). Subgingival sulcus of human oral cavity represented a major habitat of *P. gingivalis*. The pathogenesis and progression of the inflammatory events of periodontal disease was correlated with presence of *P. gingivalis* (Hajishengallis et al., 2011). In periodontitis, the number of *P. gingivalis* was increased voluminously and non-detectable or lower number in sites of plaque-associated gingivitis or with subgingival health (Schmidt et al., 2014).

Invasion of *P. gingivalis* to host cell wasrelies on the ability of bacteria to produce gingipains (a trypsin –like cysteine proteinases) which support biofilm formation and regulate host defense response (Bostanci et al., 2012). The expression of several cytokines gingipains was modulate in multi cell kinds involved: gingival fibroblasts, endothelial cells, monocytes and T cells (Palm et al., 2015; Kariu et al., 2017). Amongst the virulence factors of *P. gingivalis*, gingipains are the most important virulence factors which are responsible for damage of periodontal tissues inactivate and degrade a number of host defense and structural proteins, also it plays an essential role for *P. gingivalis* nutrient acquisition,
colonization, immune subversion and signaling (Kolenbrander et al., 2010; Mahato et al., 2016).

Initiation of periodontitis is relies mainly on embedding of microbes in subgingival dental plaque (biofilm) and interactions between microbes and host (Hajishengallis, 2015). So, this study aimed to explain the distribution of \textit{P. gingivalis} among oral cavity in patient suffering from periodontitis and to detect the correlation between biofilm formation, Fimbriae production and gingapin encoding gene.

**Methods**

A total of 150 subgingival dental plaque samples have been collected by using paper points (50mm) from patients suffering from chronic periodontitis, who visited the Teaching Hospital in the College of Dentistry in Kura University and from those who visited a Specialized Center of Dentistry in Al-Najaf Al-Ashraf city during the period from September 2016 to February 2017. All samples were collected in a proper way to avoid any possible contamination which include: cleaning of tooth surface with 70% ethanol and drying with sterile cotton swabs. Then the paper point was inputted in periodontal pockets for 1min and placed in a tube containing 1.5 ml of transport media then transferred to a laboratory for further processing.

**Isolation and characterization of \textit{P. gingivalis}**

All subgingival dental plaque samples have been cultured on blood agar supplementing with 5% sheep blood and incubated anaerobically at 37°C for 48 hr. Furthermore, a single colonies have been cultured on blood agar supplemented with 5% sheep blood, hemen (5µg/ml, Sigma, USA) and vitamin K (1 µg/ml, Sigma, USA) and incubated anaerobically at 37°C for 7 days to confirm the identification by forming black pigmented which produce by \textit{P. gingivalis} (Smalley et al., 1998). The pure colonies undergone further identification according to their microscopic features, culturing and biochemical tests (Forbes et al., 2007).

**Biofilm formation**

Biofilm screening was carried-out using microtiter plate test that was described by Kishi et al. (2012) with slight modifications as follows: A microtiter tissue culture plates (sterile, polystyrene) were overfilled with 150µl of fresh diluting (1:100) culture media [Tryptic soy broth supplemented with 1% glucose (India, Himedia)] and incubated for 24 hr. at 37°C. After incubation, the medium was tenderly removed, and the wells were washed four times with phosphate buffer saline (pH 7.2) to remove free-floating planktonic bacteria. Then the cells in plate were fixed with 2% of sodium acetate, follow by stained with 150 µL of crystal violet (BDH, England) for 45 min at room temperature and washed 5 times with D.W. One hundred and fifty microliter mixture of 33% glacial acetic acid was added to liquefy bounded crystal violet and optical density (O.D.) at 570nm was record. A correlation between adherence and biofilm production were recorded as mention by Christensen et al. (1985).

**The ability of Adherence test**

The capacity of \textit{P. gingivalis} to adhere to oral epithelial cell was detected as following steps:- 1.5×10^8 CFU/ml of bacterial isolates were prepared by diluting 72hr culture media [BHI broth, India (Himedia)] with phosphate buffer saline pH 8. The oral epithelial layers of human oral cavity were swabbing by cotton swabs for preparation of oral epithelial cells and washed three times with PBS pH 7 into sterile tubes, centrifuged at 5000 rpm/10 min and filtered. The epithelial cells were placed on a cover slide by pressing the cover on the surface of a filter paper and lifted to be dry. The cover slide was placed on a sterile glass plate then 5ml of a prepared bacterial broth was added and incubated for 1 hr at 37°C washed the cover slide with PBS to remove unglued bacteria, then the epithelial cells were fixed by ethanol for 15 minutes. After that, the slide was stained with 30% Giemsa stain (BDH, England) for 20 minutes, washed, dried and examined under light microscope (Li et al., 2004).

**PCR technique**

It was used to confirm identification of \textit{P. gingivalis} by using specific gene \textit{16S rRNA}, also the detection of \textit{fimA} genotypes (I, Ib, II, III, IV and V), \textit{kpg} and \textit{rgpA} were study. All primers were prepared from Realgene (China) as mentioned in table 1 and prepared with a final concentration 100 pmol/µl as recommended by their manufacturer’s.

**DNA extraction**

Boiling method that described by Sambrook and Russel (2001) was used for extraction of genomic DNA template from bacterial isolates. Briefly, a fresh BHI culture media was centrifuged at 6000 rpm/10min, then, the pellet was washed twice with STE buffer (0.058gm of NaCl,0.015gm of Tris base and 0.004gm of EDTA in 10 ml of distilled water) and re-suspended in STE buffer. The mixture was heated to boiling and incubated in water bath for 5min, then on ice bath for 5min. The lysate was centrifuged at 15000rpm/15min and DNA was precipitated from the supernatant by isopropanol, recovered (after 24hr of incubation in ice bath) by centrifugation at 10000rpm/10min, washed with70%
ethanol and preserved in TE buffer (BDH, England).

**Monoplex PCR technique**

It has been carried out to amplify 16S rRNA and fimA genotypes (Ib, IV and V), rgpA and Kgp. The reaction mixture (50μl) consist of: master mix (5U of i-Taq DNA Polymerase, 2.5mM for each one of dNTPs, 1X of Reaction buffer(10x) and 1X of Gel loading buffer), 2.5μl of each F and R primers, 4μl of extracted DNA template and 4μl of nuclease free water.

**Multiplex PCR technique**

It was used for amplification of fimA genotype I, II and III. The reaction mixture was prepared with final volume 50μl and consists of: master mix, 1.5μl of each F and R primers, 3μl of DNA template and appropriate volume of nuclease free water.

**Amplification condition**

PCR-Thermo Cycler (Biometra, USA) was used for amplification of target genes by using one of monoplex or multiplex PCR with specific conditions as shown in table 2.

**Agarose Gel electrophoresis**

It was carried out using 1.5% agarose gel, which prepared by dissolving 1.5gm of agarose (Prondisa, Spain) in 1X of TBE buffer (Prondisa, Spain) and staining with 10 μl of Ethidium Bromide (BDH, England) (Sambrook and Russel, 2001). The gel was Electrophoresed at 70 volt for 50min and the amplicon was visualized and photographed using Gel documentation system (Vision, Germany).

**Statistical analysis**

It was carried out using chi-squared test to analyzed a correlation between biofilm formation and fimA genotype under probability at P-value <0.05.

**Results**

**Isolation and identification of P. gingivalis**

According to the microscopic examination, culturing characteristic and biochemical test, the results showed that out of 150 subgingival dental plaque samples only 78 isolates were belonged to P. gingivalis, which appeared as a small to large colonies convex, semi mucoid, translucent after 48 hr. of incubation anaerobically and formation of black pigmented colonies after 7 days of incubation anaerobically on blood agar supplemented with 5% sheep blood, hemen and vitamin K (fig. 1-A). All isolates were negative to oxidase, catalase, methyl red and simmon citrate while it’s gave positive results to indole test and Alk/Alk without gas and H2S production on TSI agar. While the results show that only 20 isolates were belonged to P. gingivalis by using specific target gene 16SrRNAand amplify by PCR techniquewith amplicon have molecular weight 404bp (fig. 1-B).
Detection of *fim*A

As mentioned in table 3, a high prevalence of *fim*A type IV among *P. gingivalis* was observed with 20% (4 isolates) of amplicons with molecular weight 251 bp on agarose gel followed by *fim*A type III with 15% (3 isolates) and type I with 10% (2 isolates) the molecular weight of amplicons were 247 bp and 392 bp respectively (fig. 2). A low percent of prevalence was detected to *fim*A type II and V that represented 5% (1 isolates) to each one with 292 bp and 462 bp, respectively (fig. 3), while *fim*A type Ib was not detected in any isolates of *P. gingivalis*.

Detection of *rgp*A and *kgp* Genes

The results of amplification of *rgp*A and *kgp* showed that all isolates (table 4) were carry both genes with molecular weight 1700 bp and 890 bp respectively after

---

**Table 2**: Appropriate amplification conditions of *P. gingivalis* genes.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Initial Denaturation (°C/min)</th>
<th>No. of cycles</th>
<th>Condition of cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Denaturation (°C/sec)</td>
<td>Annealing (°C/sec)</td>
<td>Extension (°C/sec)</td>
</tr>
<tr>
<td>16SrRNA</td>
<td>94/2</td>
<td>40</td>
<td>94/20</td>
</tr>
<tr>
<td><em>fim</em>A(I,II,III,IV,V)</td>
<td>94/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kgp</td>
<td>94/2</td>
<td></td>
<td>94/20</td>
</tr>
<tr>
<td>Rgp</td>
<td>94/2</td>
<td></td>
<td>94/20</td>
</tr>
</tbody>
</table>

---

Fig. 1: Identification of *P. gingivalis*. A- black pigmented colonies. B- Agaros gel electrophoresis of amplicons resulted from amplification of 16S rRNA gene of *P. gingivalis* (404 bp). Lane M: DNA marker (100bp). Lane 2, 3, 5: positive results.

Fig. 2: Agarose gel electrophoresis of amplicons resulted from amplification of *P. gingivalis* *fim*A IV, *fim*A III and *fim*A I. M: DNA marker (100bp). Line (1-4): positive results to *fim*A type IV (251bp), Line (5): positive results to *fim*A type III (247 bp), Line (9): positive results to *fim*A type I (392 bp).
Detection of Biofilm formation

According to the mean of OD value at 570nm were interpreting as none, moderate and high biofilm producer, the means of OD value were <0.120, 0.120-0.240 and >0.240, respectively (Christensen et al., 1985). The results showed that all P. gingivalis isolates were biofilm producer with a high and moderate biofilm formation with percentage 75% and 25% of respectively as shown in table 4. A correlates between fimA genotype and biofilm formation was noticed in which a significant differences have been found between strong production of biofilm and genotypes of fimA that expressed by P. gingivalis whereas no significant difference was observed between moderate biofilm formation and genotypes of fimA (table 5).

Detect the adherence capacity of P. gingivalis to Oral Epithelial cells

The results of adherence capacity to oral human epithelial cells revealed that all isolates have the ability to adhere to oral epithelial cells as explained in fig. 5.

Discussion

P. gingivalis is an important bacterial etiological factor associated with periodontitis and strongly linked with chronic periodontitis, which due to its chronic continuance in the periodontium, which due to its capability to evading host immunity without obstructs the overall inflammatory response that stimulate the colonization of periodontal cavity (Kumawat et al., 2016). Characterization of P. gingivalis in most laboratories was carried out by biochemical tests, which are arduously,
Table 3: The percentage of fimA genotypes in P. gingivalis (n = 20).

<table>
<thead>
<tr>
<th>Genes</th>
<th>No. (%) of positive sample</th>
<th>Genes</th>
<th>No. (%) of positive sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>fimA I</td>
<td>2 (10)</td>
<td>fimA IV</td>
<td>4 (20)</td>
</tr>
<tr>
<td>fimA Ib</td>
<td>0 (0)</td>
<td>fimA V</td>
<td>1 (5)</td>
</tr>
<tr>
<td>fimA II</td>
<td>1 (5)</td>
<td>kgp</td>
<td>20 (100)</td>
</tr>
<tr>
<td>fimA III</td>
<td>3 (15)</td>
<td>rgp A</td>
<td>20 (100)</td>
</tr>
</tbody>
</table>

Table 4: The percentage of biofilm producer P. gingivalis (n = 20).

<table>
<thead>
<tr>
<th>Mode of biofilm production</th>
<th>Strong</th>
<th>Moderate</th>
<th>Weak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 (75%)</td>
<td>5 (25%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Table 5: A correlates between fimA genotype and biofilm formation in P. gingivalis (n=20).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Strong biofilm</th>
<th>Moderate biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td>I</td>
<td>1 (5)</td>
<td>19 (95)</td>
</tr>
<tr>
<td>II</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>III</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>IV</td>
<td>4 (20)</td>
<td>16 (80)</td>
</tr>
<tr>
<td>V</td>
<td>1 (5)</td>
<td>19 (95)</td>
</tr>
</tbody>
</table>

Chi square 2.8 0.5

Several studies confirmed the specificity and sensitivity of different type of PCR in identification of more than 70% of P. gingivalis to a species level in comparison with culturing methods that identified less than 44.4% (Al-Rawi, 2012; Kotsilkov et al., 2015; Manar et al., 2017). 16S rRNA gene (a target gene) has been broadly used in classification because it contains both genus- and species-specific regions, with low mutation rate and the patterns are well conserved (Turenne et al., 2001).

FimA (encoding by fimA) are closely responsible for many of the adhesive characteristics of the organism, binding specifically to and activation different host cells, such as human epithelial, endothelial, spleen cells and peripheral blood monocytes, resulting in liberation of cytokines and several adhesion molecules (Amano et al., 2004; Hader and Ahlam, 2016).

P. gingivalis isolates with fimA genotype IV, II and lb were a significance prevalence in chronic periodontitis than isolates with other genotype while in aggressive periodontitis, P. gingivalis genotype II was more prevalent, so that presence of fimA III genotype among P. gingivalis was most referable to increase PD, CAL and bleedings on probing (Enersen et al., 2008). P. gingivalis that possess for fimA genotype IV were found to be considerably related with chronic periodontitis, while presence of P. gingivalis, which possess a combination of fimA genotypes IV/V and I/IV were associated with periodontal diseases, whereas no disease association was appeared with strains of fimA genotype II because of their recurrently presence at healthy sites (Hayashi et al., 2012; Griffen et al., 1999). A virulent P. gingivalis fimA type I genotype appeared in a high pervasion among chronic gingivitis patients whereas its existence was low in chronic periodontitis, which signifies its reversible condition (Krishnan et al., 2016).

P. gingivalis secretes proteolytic gingipains (Kgp and RgpA/B) as zymogens inhibited by a pro-domain that is removed during extracellular activation and its bind either directly to extracellular matrix proteins due to the adhesin function or indirectly contributes to bacterial adhesion by processings the fimbriillin subunit (Pomowski et al., 2017). It’s essential for P. gingivalis survival in vivo and in experimental infection induction. P. gingivalis that harbor gingipains have been implicated in periodontal pathogenesis by inhibiting inflammation resolution and is linked with systemic chronic...
inflammatory conditions (Castro et al., 2017).

A correlation between gingipains and fimA genotypes has been found in which all isolates that possess gingipains were possess one or more than one type of fimA genotypes, which may due to the many factors that stimulate the expressiveness of virulence factors (including proteolytic enzyme such as gingipains) such as environmental conditions, variation in chromosomal genes or mutational analyzing of fimbriae -, gingipain -, and/or hemagglutinin-associated genes which detect a non-fimbrial co-aggregation system that relies on translation molecules of rgpA, rgpB, kgp and hagA genes in P. gingivalis (Abe et al., 2004). Cysteine proteinases have formerly been found to play a significant role in P. gingivalis biofilm formation and invasion of host cells by processing’s precursors fimbriae and promote adhesion as well as facilitate the initial attachment of P. gingivalis to surfaces and host cells including gingival epithelial cells particularly Rgp, which is essential in the formation of microcolonies and in controlling the biovolume and play essential role in supply of precursor fimbriae and Kgp while Kgp was required to release or transmit of Rgp into the surroundings (Chen and Duncan, 2004).

Biofilms formation which mediated largely through FimA plays significant roles in human infections and diseases. It was estimated that 65–80% of the microbial infections are caused by bacteria adhered to surfaces (Davies, 2003). P. gingivalis was ableto express a number of adhesins correlated with either the outer membrane or biofilm that enhancement its adhesion to tooth surfaces, gingival epithelial cells, basement membrane components, erythrocytes and oral bacteria (Choi et al., 2016).

The ability of P. gingivalis to produce biofilm were effected by several factors such as the cell surface hydrophobicity, gingipains, haemagglutinin, capsule and other types of fimbriae (Amano, 2010; Bostanci et al., 2012). Fimbria and gingipains seems to operate coordinately to regulate the developing of mature P. gingivalis biofilms where FimA fimbriae catalyzes initial biofilm formation and exert a resuming regulation on biofilm maturation while Mfa and Kgp have a regulatory and suppressive role during biofilm development whereas Rgp dominates the morphology and biovolume of microcolony (Nagano et al., 2013).

In patients with severe periodontitis, a high oral load of P. gingivalis with incessant inflammation could impact and supports the immune evasion of oral carcinomas (Zandberg and Strome, 2014). P. gingivalis is capable to internalizing and re-program the immune signaling pathways in host cellsas a facultative intracellular bacterium (Irshad et al., 2012). Alongside the invasive possibility of P. gingivalis, it also sheds membrane vesicle to aquire functional virtues in immune evasion such as OMVs which contains the components of the outer membrane including LPS, muramidic acid, capsule, fimbriae and gingipains (Groeger et al., 2017). P. gingivalis membrane vesicles may affectuate cellular responses associating in adenitis and initiating of acquired immunity, so, inducing anti-P. gingivalis local (mucosal) immunity in the oral cavity in addition to systemic immune responses follows immunization would be an effectual strategy for the protecting against P. gingivalis infection (Shimizu et al., 2017). Also, enhancement of bacterial adhesion/invasion of epithelial cells was results from propagation of P. gingivalis mutants where fimA genotype I was replace by fimA genotype II while diminished efficiency was results from substitution of fimA genotype II with fimA genotype I (Kato et al., 2007).

Conclusion

P. gingivalis associated with periodontitis were possess many virulence factors like fimA (where fimA genotype IV was most predominant), cysteine proteinase (represented by kgp and rpg), biofilm formation and their ability to adherence to human oral epithelial cells. Also, a significance differences has been found between strong biofilm formation and fimA genotypes.

References


Genotypic and Phenotypic Detection of some Virulence Factors among \textit{P. gingivalis}


