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GENOTYPIC AND PHENOTYPIC DETECTION OF SOME VIRULENCE FACTORS AMONG *PORPHYROMONAS GINGIVALIS* RELATED WITH PERIOD ONTITIS IN AL-NAJAF AL-ASHRAF CITY, IRAQ

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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 gingapin encoding genes. Isolation and identification of *P.gingivalis*frompatients suffering from chronic periodontitis (CP) according to phenotypic detectable of biofilm formation and the capacity of bacterial cells to adherence 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 belonged to *P. gingivalis*. Genotypic detection of *fim*A genotypes revealed that *fim*A type IV gene was the most predominance (20%) followed by *fim*A type III while *fim*A type II and V show up the lowest percentage (5%) whereas *fim*A type Ib was not identified. Genetic investigation of *kgp* and *rpg*A 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 *rpg*. A correlation has been found between biofilmformation, adherence to epithelial cells and possessing of *fimA* genotypes.

Key words : *P. gingivalis*, *fim*A genotypes, periodontitis, PCR.

Introduction

Porphyromonas gingivalis is an opportunistic pathogen associated withup 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,

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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 *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 1mint and placed in a tube containing 1.5 ml of transport media then transferred to a laboratory for further processing.

Isolation and characterization of *P. gingivalis*

All subgingival dental plaque samples have been cultured on blood agar supplementing with 5% sheep blood and incubated anaerobically at 37ºC 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 37ºC for 7 days to confirm the identification by forming black pigmented which produce by *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 wellswere 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 µLof 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 *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 7into 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 37ºC 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 *P. gingivalis* by using specific gene *16S rRNA,* also the detection of *fimA* genotypes (I, Ib, II, III, IV and V), *kgp* and *rgp*A 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%

Table 1 : The oligonucleotide sequences.

Genes	Oligonucleotide Sequence 5' ⟶ 3'	Molecular Weight of Amplicons	References
16SrRNA	F AGG CAG CTT GCC ATA CTG CG R ACT GTT AGC AAC TAC CGA TGT	404bp	(Amano *et al*., 1999)
*fim*A type *I*	F CTGTGTGTTTATGGCAAACTTC R AACCCCGCTCCCTGTATTCCGA	392bp	
*fim*A type II	F GCATGATGGTACTCCTTTGA R CTGACCAACGAGAACCCACT	292bp	(Moon *et al*., 2011)
*fim*A type III	F ATTACACCTACACAGGTGAGGC R AACCCCGCTCCCTGTATTCCGA	247bp	(Amano *et al*., 1999)
*fim*A type IV	F CTATTCAGGTGCTATTACCCAA R AACCCCGCTCCCTGTATTCCGA	251bp	
*fim*A type V	F AACAACAGTCTCCTTGACAGTG R TATTGGGGGTCGAACGTTACTGTC	462bp	(Nakagawa *et al*., 2000)
*fim*Atype Ib	F CAGCAGAGCCAAAAACAATCG R TGTCAGATAATTAGCGTCTGC	271bp	(Nakagawa *et al*., 2002)
*rgp*A	F AGT GAG CGA AAC TTC GGA GC R GGTATCACTGGGTATAACCTGTCC	1700bp	(Allaker *et. al*., 1997)
Kgp	F GAA CTG ACG AAC ATC ATT G R GCT GGC ATT AGC AAC ACC TG	890bp	(Beikler *et al*., 2003)

ethanol and preserved in TE buffer (BDH, England).

Monoplex PCR technique

It has been carried out to amplify *16S rRNA* and *fim*A genotypes (Ib, IV and V), *rgp*A 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 *fim*A 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 largecolonies 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 H_2S production on TSI agar. While the results show that only 20 isolates were belonged to *P. gingivalis* byusing specific target gene *16SrRNA*and amplify by PCR techniquewith amplicon have molecular weight 404bp (fig. 1-B).

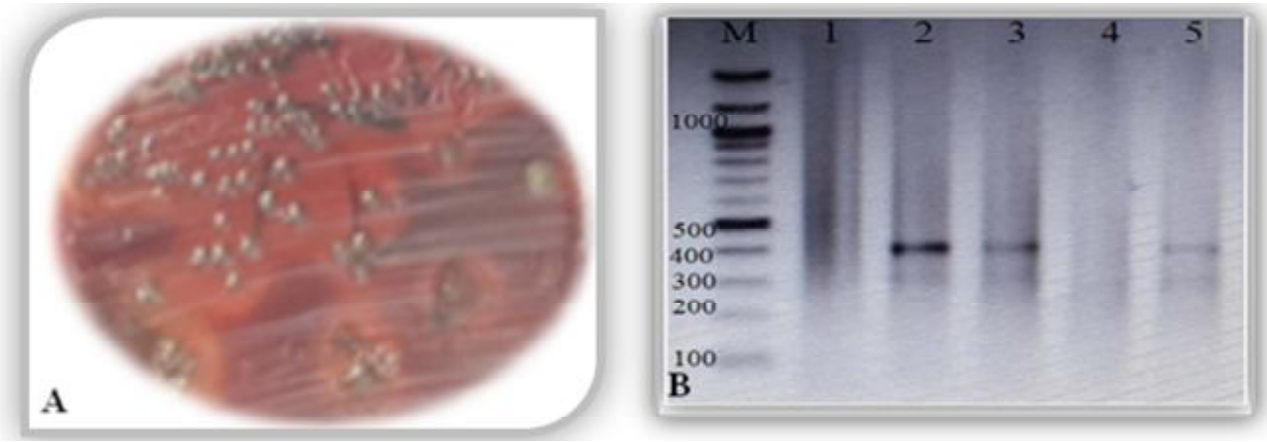

Fig. 1 : Identification of *P. gingivalis*. A- black pigmented colonies. **B-** Agaros gel electrophoresis of amplicons resulted from amplification of*16S rRNA* gene of *P. gingivalis* (404bp). Lane M: DNA marker (100bp). Lane2, 3, 5: positive results.

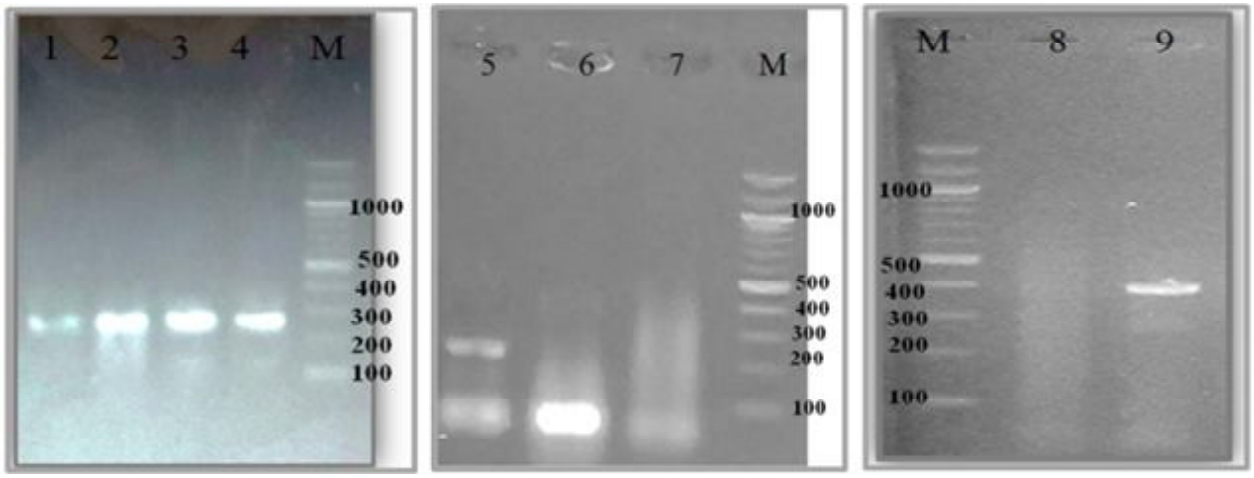

Fig. 2 : Agarose gel electrophoresis of amplicons resulted from amplification of *P. gingivalis fim*A IV, *fim*A III and *fim*A I. M: DNA marker (100bp) Line (1-4): positive results to *fim*A type IV (251bp), Line (5): positive results to *fim*A type III (247 bp), Line (9): positive results to *fim*A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

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

Genes	Initial Denaturation (ºC/min)	No. of cycles	Condition of cycle			
			Denaturation (ºC/sec)	Annealing (ºC/sec)	Extension (ºC/sec)	Final extrnsion (ºC/min)
16SrRNA	94/2	40	94/20	65/10	72/25	72/2
*fim*A*(I,Ib,II,III,IV,V)*						
Kgp	94/2		94/20	65/10	72/45	72/3
Rgp	94/2		94/20	65/20	72/120	72/4

Detection of *fim*A

As mentioned in table 3, a high prevalence of *fim*A type IV among *P. gingivalis* was observedwith 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 247bp 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. gingivali*.

Detection of *rgpA* 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

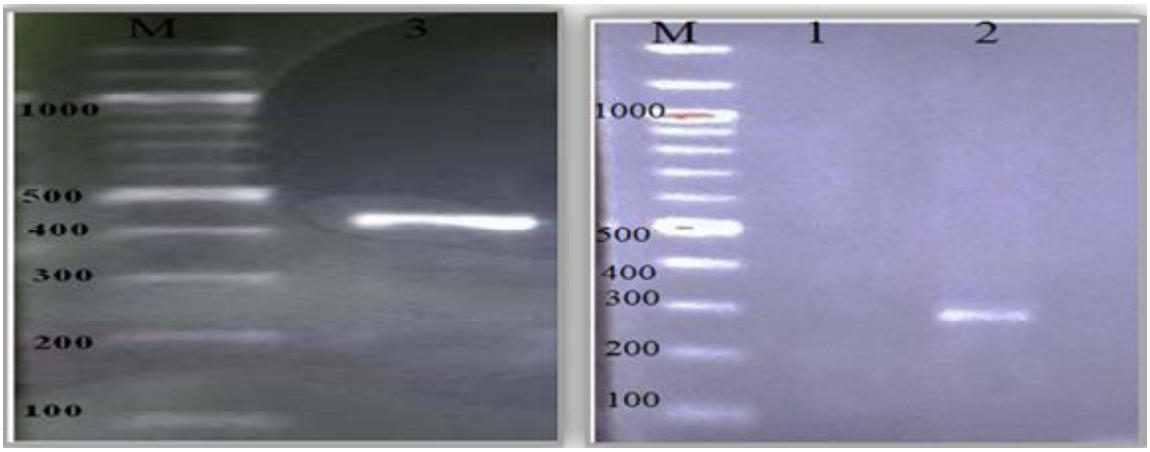

Fig. 3 : Agarose gel electrophoresis of amplicons resulted from amplification of *P. gingivalis fim*A II and *fim*A V. M: DNA marker (100bp) Line (2): positive results to *fim*A type II (292bp), Line (V): positive results to *fim*A type V (462 bp).

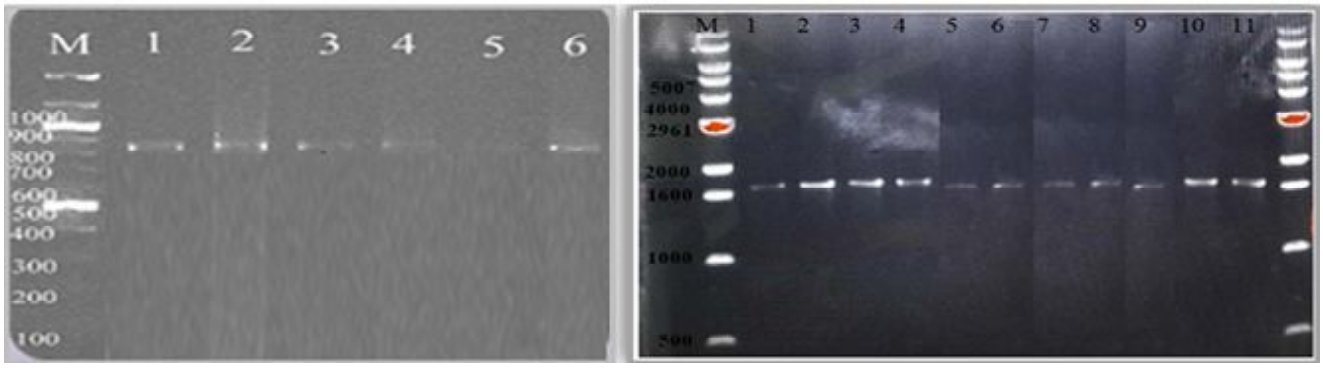

Fig. 4 : Agaros gel electrophoresis of amplicons resulted from amplification of *P.gingivalis* gingapain. A- *kgp* amplicon with molecular weight (890bp). Lane M:DNA marker(500bp) Lane1-6: positive resultes. B- *rgp*A amplicon with molecular weight (1,700bp). Lane M:DNA marker(1kb) Lane 1-11: positive results.

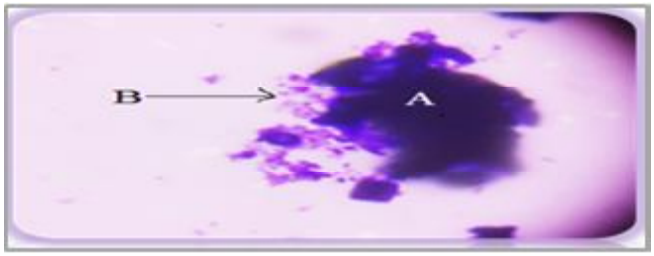

Fig. 5 : The ability of *P.gingivalis* to Adherence to oral human epithelial cells. A: Oral human epithelial cells. B: *P. gingivalis*.

pouring on agarose gel (figs. 4A and 4B).

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 *fim*A genotypes in *P. gingivalis* (n =20).

Genes	No. (%) of positive sample	Genes	No. (%) of positive sample
*fim*A I	2 (10)	*fim*A IV	4 (20)
*fim*A Ib	0 (0)	*fim*A V	1(5)
*fim*A II	1(5)	*kgp*	20 (100)
*fim*A III	3 (15)	*rgp* A	20 (100)

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

Mode of biofilm production		
Strong	**Moderate**	**Weak**
15 (75%)	5 (25%)	0 (0%)

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

Genotypes	Strong biofilm		Moderate biofilm	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
I	1(5)	19(95)	1(5)	19(95)
II	0(0)	20(100)	0(0)	20(100)
III	0(0)	20(100)	1(5)	19(95)
IV	4(20)	16(80)	0(0)	20(100)
V	1(5)	19(95)	0(0)	20(100)
Chi square	**2.8**		**0.5**	

time expendable, cumbered and unreliable while bacterial culture being the gold standard for the growing and characterization of *P. gingivalis*. The accurateness and propriety of the microbiological analyzing results were also influenced by its capability to dependable measurement the presence and frequentness of bacteria in collected samples (Vajawat *et al*., 2013).

Cellular pigmentations were a significant virulence factor of the oral *P. gingivalis*. Pigmentations has been linked with many bacterial functions involved (but not limited): colonization, conseving a local anaerobic environment by encasement oxygen molecules and defending against reactive oxygen species (ROS) produce by immune cells (Klein *et al*., 2017). The results of this study refer to the specificity and sensitivity of PCR in comparison with the conventional method (culturing characteristic and biochemical test) in the identification of *P.gingivalis*, which may due to limiteds viability of *Porphyromonas* exterior anaerobic habitats and losing of pathogen during cultivation or because of transporting of the specimens from patient to a microbiological laboratory with a specific challenging due to inability to keep pathogen in a viable count required for a standard cultivation (AL-Rubiae *et al*., 2015). 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 *fim*A) 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 *fim*A genotype IV, II and Ib 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 *fim*A 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 *fim*A 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 fimbrillin 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 *fim*A 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 *fim*briae -, 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 dominatesthe 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, muramic 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* genotyps.

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