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Bacteriological Study of Pigmented Bacteria and Molecular Study Based for 16s rRNA Gene.

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ABSTRACT

Periodontitis is an infectious disease leading to the damage of periodontium. The etiology of this disease is microorganisms deposits play an essential role in the pathogenesis. The study aimed to isolation and to identification of *P. gingivalis*, using The PCR technique based on *16s rRNA gene* to confirm of identifications. The ability of this bacterium to adhere to epithelial cells of oral cavity and evaluating the effect of some extracts on biofilm formation. Eighty clinical sample was collected from subgingival plaque of dental of patients with (50) chronic and (30) aggressive periodontitis. The patient was admitted to Babylon University (teaching Hospital of college of Dentistry). The samples of plaque were subjected to different methods for identification of *P. gingivalis*. It was found that twenty two of *P. gingivalis* isolates were recovered. Eighteen isolates obtained from (CP) &four isolates from (AP). Twelve isolates of *P.gingivalis* from twenty two were detected by method of molecular focusing on the role of 16s rRNA gene for this microorganism. Ten species of bacterial isolates was isolated from chronic periodontitis, and two bacterial isolates were isolated from aggressive periodontitis subgingival plaques. All bacterial isolates were investigated to detect Attachment of *P.gingivalis* to epithelial cells oral cavity. The result showed that all isolates have ability to attach to epithelial cells oral cavity. The study also detected the ability of the bacteria for production of gingipain; it was found that all tested isolates were positive for this enzyme at a rate 100%. In addition, biofilm formations were tested in the semi quantitative of test of microtiter plate. The results of this study revealed that the isolates were biofilm former, high and moderate biofilm formation mode were accounted for (50%) while there are no isolates that express non biofilm formation. The study was also evaluating the effect of some extracts on biofilm formation. The higher effects for Alum of the bacteria were showed followed by *Clove*, in contrast to lowest effect of *C. rotundus*. *P. gingivalis* were the well-recognized potential period on to pathogen. The PCR technique based of *16s rRNA gene* is sensitive and bacterial culture being the gold standard of the growth and identification of P.*gingivalis*. all isolates able to produce biofilm. *Alum*, *Clove,* and *C.rotundas* have an effect on *P. Gingivalis* biofilm.

Keywords: *P. gingivalis*, periodontitis, 16s RNA gene

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INTRUDUCTION

Periodontitis is an infectious disease leading to the damage of periodontium. The etiology of the disease is bacterial deposits play an essential role in the pathogenesis. The predominantly of gram negative obliged anaerobic bacilli is involved in periodontitis and accumulate in the subgingival plaque. *P. gingivalis* has been responsible for chronic periodontitis [1].

P.gingivalis is gram negative obliged anaerobic bacilli that produce porphyrin black pigments on blood agar. In this study concentrated and focused on genetic primers which found that P. gingivalis detection could be achieved with varying level of success with specific primers for 16s rRNA -encoding genes. The rRNA sequences of P. gingivalis show equal 95% homology. Moreover, these genes are ideal targets for unique identification of P. gingivalis [2]. In addition, this genetic marker can detect P. gingivalis from different positions and in a study done on periodontal infections focused on this gene as genetic marker with high detection rate of P. gingivalis as the study detected this bacterium in a rate of one hindered present from subgingival sulks and from endodontic infection [3]. Morillo et al., (2004) [4] found that a PCR-based on 16s rRNA -encoding genes had a specificity of 96% and a sensitivity of 98.5% compared with culture media from periodontitis infection.

The fimbriae virulence of P. gingivalis is heavily associated with virulence factors in adhesion, adherence, invasion and colonization. It has short fimbriae, long fimbriae, and other accessory components, each of which has distinct functions [5]. Major fimbriae (FimA) are long filamentous, peritrichous[6]. They has a role in attachment of biofilms, organization and act as adhesins that mediate colonization and invasion of the cellsof host due to P. gingivalis virulence (Kubinowaet al., 2008). Minor fimbriae (Mfa1) is essential for cell-cell auto aggregation, adhesion with other dental commensals and recruitment for colony formation and induce production of many cytokines from macrophages that in turn can induce alveolar bone resorption.

Accessory fimbriaeare Fim C, D, and E accessory part associate with the main Fim A protein has a role in binding or adherence with matrix proteins and interaction with CXC-chemokine receptor four [7].

Gingipains are cysteine proteinases that are responsible for the virulence of P. gingivalis .
It produces a wide range of enzymes; the Arg and Lys specific extracellular cysteine proteinases can damge serum proteins including complement factors and immunoglobulin as well as extracellular matrix proteins (e.g. fibrinogen, laminin) and activate cytokines (e.g. TNF and IL-6).

Gingipain is very important in virulence factors of the periodontal infection and functions related to cell host colonization and invasion. These gingipains serve many functions for the microorganism, which contributed to the survival and virulence[8].

Biofilm is an accumulation extracellular product of organisms and forming a community on a surface [9]. The biofilm Dental plaque is the complex community of microorganisms formed on the teeth surfaces, embedded in an extracellular matrix of polymers of host cell and bacterial origin. Clinically, the dental plaque is the soft, tenacious deposit that forms on teeth. [10].A major advantage of biofilm is protection from detrimental factors such as the factors of host defense as well as antimicrobial substances including antibiotics. It can facilitate the processing and uptake of the nutrients, cross-feeding, removal potentially harmful metabolic products and development adaptive environment such as reduced reduction oxidation potential [11]. The aims of this study are to isolation and to identification of P.

gingivalis bacteria, using The PCR technique based on 16s rRNA gene for confirm of identifications. The ability of this bacterium to attach to epithelial cells oral cavity and evaluating the effect of some extracts on biofilm formation. Also the production of gingipain from bacteria.

METHODOLOGY

Bacteriological Study

Isolation and all biochemical tests for diagnosis of *Porphyromonas gingivalis* was done according to Forbes et al., 2007 and MacFaddin (2000) [12].

Molecular detection based on16s rRNA -encoding genes for diagnosis of *P. gingivalis* was done according to Vajawat et al., (2013) [13].

Adherence Ability Test:

The ability of *P. gingivalis* to adhere to epithelial cell of oral cavity is one of important virulence properties of these bacteria and detected (Avila-Compose *et al*., 2000) [14].

Extracellular Protease Production Test was done according to Piret *et al.*, (1983) [15].

Biofilm Production: Tissue culture plate method (TCP):

Tissue culture plate method (TCP) assay (biofilm assay) (also called semi quantitative microtiter plate test described by Christensen *et al*., (1985) and Mathur *et al* (2006) [16-17].

Effect of extracts and Alum 50 % on Biofilm Formation:

The same procedure described in (method of tissue culture plate for detection biofilm formation) was done with modification. In vitro Antipigment Activity of Flagyl Solution was done according to Hadi et al., 2013) [18].

RESULTS

Isolation and Identification of Porphyromonas gingivalis Isolates:

A total of 103 clinical samples were collected from sub gingival dental plaques from patients with 72 chronic and 31 aggressive periodontitis.

The patient was admitted to Babylon University (Hospital of teaching in college of Dentistry) in Hilla city, during the period from February 2013 to June 2014. It was found that 23 outof P. gingivalis were recovered by using selective media where 26.3% (19) bacterial isolates obtained from (CP) and 12.5% (4) isolates from (AP). The Characterization of P. gingivalis isolates: The identification of P. gingivalis isolates depends mainly on the cultural and biochemical test and also microscopic patterns.

The result in table (1) demonstrates that *P. gingivalis* is an anaerobic, G-ve. It is observed to be non-motile and small coccobacilli. When colonized on blood agar it forms black spots, black- pigmented colonies, due to it takes part in Iron Transport, the way it does this is by using a hemin as a device to help it transport iron. The organisms are single cell, non-spore forming, catalase negative, hadn't able to ferment some sugars such as glucose, lactose, fructose, mannitol, and mannose *P. gingivalis* is cultured on selective media (P.GING), it is an enriched selective medium for the isolation and presumptive identification of *P. gingivalis*, which consist of Columbia agar base 42.5 g is supplemented with sheep blood 5.0 ml, bacitracin, 10.0 mg, colistin 15.37 mg and nalidixic acid 15.0 mg as selective agents for the isolation of *P. gingivalis* (NCCLs, 2004), Alencar et al ., (2016) [19-20].

Confirmed Detection of *P. gingivalis* by PCR Using Specific Primers

Twelve bacterial isolates of P. gingivalis out of twenty three were detected by molecular method focusing on the role of 16s rRNA gene of P. gingivalis (table 1) .

Eight isolates was isolated from subgingival plaque of CP, and four isolates were isolated from AP subgingival plaque. The specificity of primers of the P.gingivalis was used to amplify 404- bp fragment of the 16s rRNA gene was detected with various oral and non-oral organisms (figure 1).

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Detection of virulence factors in *P. gingivalis*:

Detection adherence ability of *P. gingivalis* to epithelial cells of oral cavity:

Adherence of *P. gingivalis* to epithelial cells of oral cavity is the first step in the pathogenesis of *P. gingivalis* infection and is facilitated by the action of several adhesions located on the surface of bacteria (table 2).

Detection of Gingipain in *P. gingivalis*:

Gingipain is a potential virulence factor in *P. gingivalis* so it was investigated in 12 *P. gingivalis* isolated from different types of periodontitis. In present study extracellular protease activity was phenotypically detected in M9 media (supplemented with 20% glucose and 1% casein) and it was found that all isolate was positive to this enzyme at a rate 100%. The extracts of plant (50 % concentrations) have activity on biofilm formation.

The effect of some plant extracts on biofilm formation. The results showed that using of these extracts at (50 %) concentrations have some effect on biofilm formation. From present results in table (3), the biofilm formation affected by using Clove, Alum and *C. rotundus* at (50%) concentrations, respectively. According to the effects of Clove (50%), the percentage of biofilm formation changed from (100%) to (50%) ,while Alum (50%) showed high percentage of change from (100%) to (25%),in contrast *C. rotundus* (50%) showed lowest percentage of change from (100%) to (75%).

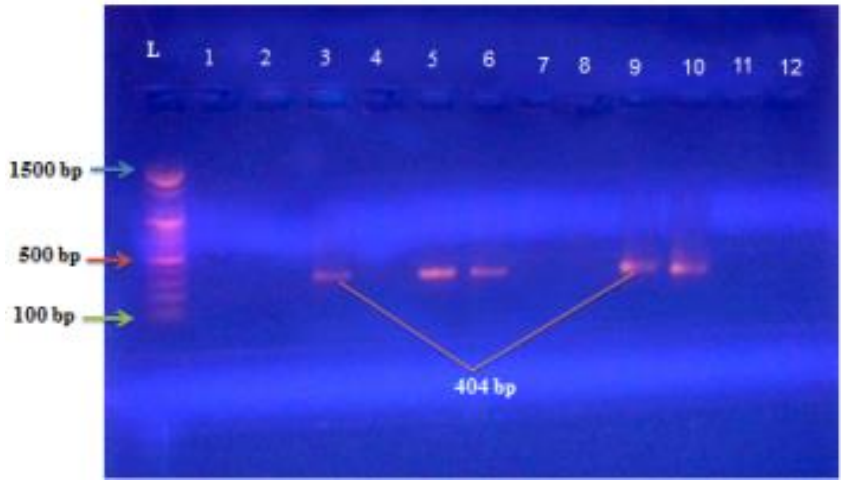

Figure (1) Gel electrophoresis of PCR product of *P. gingivalis16s rRNA* gene. Lane of isolates numbered (3, 5, 6, 9, 10) were positive

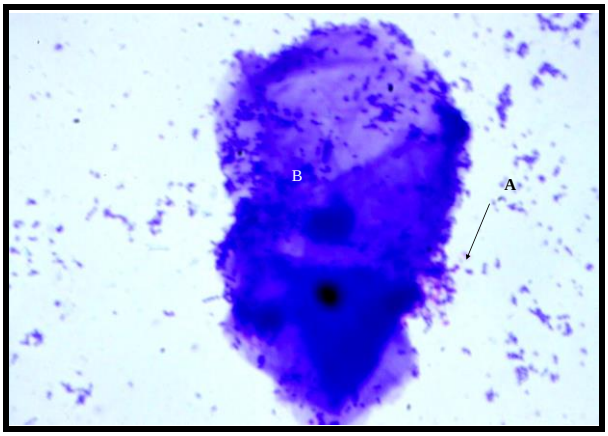

Figure (2) Adherence ability of *P. gingivalis* to epithelial cells of oral cavity.

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Table (1): Biochemical Tests and the Microscopic Examination of *P. gingivalis*

Tests	Results
Growth on blood agar anaerobic condition	Black-pigmented colonies
Gram stain reaction	G-ve, coccobacillli
Indole	+ve
Growth on selective media (P.GING)	+ve
Catalase	-ve
Motility	-ve
Nalidixic acid, Colistin and Bacitracin requirement	+ve
sugar fermentation	-ve
Vancomycin sensitivity	+ve

Table (2) Production of biofilm in *P. gingivalis*

Bacterial isolate No.	Biofilm			
	Strong	Moderate	% of biofilm formation	Weak
P. gingivalis (4)	2	2	100 %	0

Table (3): Effect of Clove, Alum potassium phosphate and *Cyperus rotundus* (50 %) on biofilm formation

Bacterial isolate (No.)	Biofilm after adding Clove (50 %)			
	Strong	Moderate	% of biofilm formation	Weak
P. gingivalis (4)	0	2	50 %	2(50%)
	Biofilm after adding Alum (50 %)			
	0	1	25%	3(75%)
	Biofilm after adding *Cyperus rotundus* (50 %)			
	1	2	75%	1(25%)

DISCUSSION

Clinical samples (103) were collected from dental plaques (sub gingival) from patients with aggressive (31) and chronic periodontitis (72). It was found that 22 bacterial isolates were isolated by selective media where chronic periodontitis eighteen (26.3%) isolates and four isolates (12.5%) from aggressive. *P. gingivalis* is involved in the destruction of human tissue and therefore may play a mechanistic role in AP-related autoimmunity [21].

The identification of *P. gingivalis* isolates depends mainly on the cultural and biochemical characteristics and also microscopic patterns. The result in table (1) demonstrates that *P. gingivalis* is an

anaerobic, G-ve. It is observed to be non-motile and small coccobacilli. When colonized on blood agar it forms black spots, black- pigmented colonies, due to it takes part in Iron Transport, and the way it does this is by using a hemin as a device to help it transport iron. The black pigmentation that is detected by Al-Khafagee *et al.,* (2013) to builds up the iron. Catalase negative, hadn't able to ferment some sugars such as glucose, lactose, fructose, mannitol, and mannose [22]. Tryptophan is an amino acid that can be oxidized by some bacteria to form three major end products: Indole, pyruvic acid, and ammonia [12].

Furthermore, *P. gingivalis* was showed 100% sensitivity to vancomycin, which can provide diagnostic features for this bacterium [23]. Motility test was done for all isolates by the use of motility media and compared with motile bacteria as a positive control.

Confirmed Detection of *P. gingivalis* by PCR Using Specific Primers:

A twelve isolates of P. gingivalis from twenty two were detected by molecular method focusing on the role of 16s rRNA gene of P. gingivalis. Ten isolates was isolated from subgingival plaque of CP, and two isolates were isolated from AP. the specificity of the P. gingivalis primers were used to amplify 404- bp fragment of the 16s rRNA gene was detected with many oral and non-oral organism (figure 1) [24]. The primers specific for black bacteria specifically amplified P. gingivalis DNA, while PCR product was not obtained with any of the other organisms tested and observed that quantification of DNA from P. gingivalis was not affected when DNA from many of other species was present in the PCR mixture [25]. Study by Sakamoto et al., (2005) [26]. concentrated and focused on this primers and found that P. gingivalis detection and achieved with varying degree of success with specific primers for 16s rRNA -encoding genes yet, the rRNA sequences of P. gingivalis show equal 95% homology. These genes were ideal targets for unique and confirm identification of P. gingivalis.

In addition to that, this genetic marker detect the P. gingivalis from many sites on periodontal infections concentrated on specific genes as marker of genetic with high detection percent of P. gingivalis as the study detected this organism in a rate of one hindered present from subgingival sulcus and from endodontic infection [27].

Detection of virulence factors in *P. gingivalis*:

Detection adherence ability of *P. gingivalis* to Epithelial Cells of Oral Cavity:

Adherence of *P. gingivalis* to epithelial cells of oral cavity is the first step in the pathogenesis of *P. gingivalis* infection and is facilitated by the action of several adhesions located on the surface of bacteria. The result showed that all *P. gingivalis* isolates (12) have ability to adhere to epithelial cells of oral cavity (figure 2).

The high adherence ability correlated with study done by Enersen *et al.*,(2008) [28], they focused on the most essential role of fimbriae in the binding ability to cell of host including the epithelial cells of oral cavity. Especially, the binding activity to epithelial cells of oral cavity can be the critical step in its invasion and survival in host gingival tissues and thus contribute to enhance the pathogenicity of this organism.

The roles of surface components of P. gingivalis like fimbriae in have contact with oral cavity tissues and cells due to of components of *P. gingivalis were* potentially important in the occurrence of periodontal diseases [29]. Some studies on *P. gingivalis* fimbriae demonstrated the importance of it in mediation of interaction of this bacterial organism with oral cavity tissues and interrupt the cellular signaling via extracellular matrix proteins/ integrins in periodontal regions [30].

Detection of Gingipain in *P. gingivalis*:

Gingipain is a potential virulence factor in *P. gingivalis* so it was investigated in 12 *P. gingivalis* isolated from different types of periodontitis**.** In present study extracellular protease activity was phenotypically detected in M_9 media(supplemented with 20% glucose and 1% casein) and it was found that all isolates of were positive for this enzyme at a rate 100%. These results ware correlated with that other study obtained by Sheets *et al.,* (2012) [31].

The role of gingipain is that it contributes to pathogenesis through manipulation of the host immune response as target several components of the host innate immune system, including complement, antimicrobial peptides, cytokines [32].

The benefit to the organism of the production of extracellular proteases enzyme can be viewed in terms of the development of another sites for colonization within the host, and the acquisition of carbon/nitrogen sources and micronutrients for catabolic and anabolic purposes and for evasion of the host defenses [33].

Biofilm formation

The Biofilm formations of polymeric surfaces were tested in methods of the semi quantitative microtiter plate (biofilm assay) test using broth (Trypticase Soy Broth) supplemented with one present from glucose. This assay was repeated as triplicate to increase the accuracy of assy.

Regarding the OD value at 630 nm the results were interpreted as none, moderate and high biofilm former when the mean of OD value were (<0.120, 0.120-0.240, and >0.240) respectively. The results found that all isolates of P. gingivalis were biofilm former, high and moderate biofilm formation mode were account for (50%) while there are no microbial isolates that express non biofilm formation, (table 2) the virulence factors of Biofilm to facilitates attachment of microorganisms to biomedical surfaces. The protect them from host antimicrobial therapy and immune response [34].furthermore, the production of biofilm was promote the colonization and lead to increased rate of periodontitis and such infections may be difficult to treated as they exhibit multidrug resistance.

The biofilm of cell has been shown to be significant resistant to antiseptic than planktonic cells. For example, many persistent and chronic bacterial infections, including, periapical abscesses, periodontal infections and dental caries, are now believed to be linked to the formation of biofilms [35].

The effect of some extracts on biofilm formation was studied. The results of extracts showed that using of the extracts have activity on biofilm formation. From present results in table (3), the biofilm formation affected by using Clove, Alum and *C. rotundus* at (50%) concentrations, respectively.

According to the effects of Clove (50%), the percentage of biofilm formation changed from (100%) to (50%) ,while Alum (50%) showed high percentage of change from (100%) to (25%),in contrast *C. rotundus* (50%) showed lowest percentage of change from (100%) to (75%).

These results agreed with (Kothiwale et al., 2014) [36] who cleared that the uses of many types of extract as newly formulated mouthrinse containing, clove, and alum demonstrates antimicrobial, antigingivitis, and antiplaque properties, which may be used as an adjuvant to therapy of periodontal in the treatment and prevention of periodontal diseases.

CONCLUSION

The bacterium is a well-recognized potential period on to pathogen. The technique of PCR based on *16s rRNA gene* is sensitive and bacterial culture being the growth and identification of P.*gingivalis*. all isolates able to produce biofilm.*Alum*, *Clove,* and *C.rotundas* have an effect on *P. Gingivalis* biofilm.

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