Distribution of *Granulicatella adiacens* and *Porphyromonas gingivalis* among ortho and non-orthodontic Patients with Gingivitis in Kufa City /Iraq.

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الخلاصة:
هدفت هذه الدراسة إلى التعرف على توزيع غرانوليكاتيلا أدياسنس و بورفيروموناس لينغيفيك ودور أسلاك تقويم الأسنان على نمط مقاومة المضادات الحيوية من العزلات البكتيرية. تم جمع ما مجموعه 78 عينة مسحة اللثة من المريض مع أسلاك تقويم الأسنان الذين يعانون من التهاب اللثة و 71 عينات تم جمعها من صحية دون أسلاك تقويم الأسنان خلال أربعة أشهر من عيادات الأسنان الخاصة.
اظهرت نتائج العزلة والتعرف على العزلات البكتيرية باستخدام الاستزراع والاختبارات البيوكيميائية التقليدية وكذلك التقنية الجزيئية باستخدام ال S16 الريباسي للكشف عن ال G. أدياسنس والعنصر التسلسلي IS1126 للكشف عن P. لينجيفاليس ان 54 عزلة (29.6٪) كانت تنتمي إلى G أدياسنس و 4 العزلات تنتمي إلى P اللثة.
لشرح دور أسلاك تقويم الأسنان على العزلات البكتيرية تم إجراء تجربة طفرة. وأظهرت النتائج نفس التغيير الذي تم الحصول عليه عند استخدام كل من نيتي والأسلاك الفولاذ المقاوم للصدأ على G. أدياسنس و P. اللثة بعد hr-96hr24 من الحضانة. أظهر نمط مقاومة المضادات الحيوية للعزلات الأصلية والمعالجة مع نيتي والفولاذ المقاوم للصدأ زيادة في مقاومة المضادات الحيوية لباسيتراسين، سيفتازيديم،
أوجمنتين، والاريثروميسين في حين تبقى المضادات الحيوية الأخرى حساسة مثل سيفوتاكسيم و أميكاسين لجميع العزلات.

Abstract
This study aimed to investigate the distribution of *Granulicatella adiacens* and *Porphyromonas gingivalis* and the role of orthodontic wire on antibiotic resistance pattern of bacterial isolates. A total of 78 gingival swab samples have been collected from patient with orthodontic wires suffering from gingivitis and 71 samples were collected from healthy without orthodontic wire during four months from private dental clinics.

The results of isolation and identification of bacterial isolates by using culture and conventional biochemical tests as well as molecular technique using *16S rRNA* for detection of *G. adiacens* and insertion seqence element *IS1126* for detection of *P. gingivalis* showed that 54 (29.6%) isolates were belong to *G adiacens* and 4 isolates were belong to *P gingivalis*.

To explain the role of orthodontic wires on bacterial isolates a mutation experiment was carried out. The result showed the same change has been obtained when using both NiTi and stainless steel wire on *G. adiacens* and *P. gingivalis* after 24hr-96hr of incubation. Antibiotic resistance pattern of both original and treated isolates with NiTi and stainless steel showed increased in antibiotic resistance to bacitracin, ceftazidim, ogmentin, and erythromycin while other antibiotic remain sensitive such as cefotaxim and amikacin for all isolates.

Introduction

periodontal disease refers to gingivitis and periodontitis is a reversible inflammation induced by dental plaque of the gingiva (Suvan *et al.*, 2011), while periodontitis is a microbial inflammatory condition of the gingivae causing destruction of ligament and alveolar bone supporting the teeth resulting in oral malodor and loss of tooth and then loss the quality of life (Al-Harthi *et al.*, 2013). There are more than 300 species identified in the oral cavity, only small

group of gram-negative organisms which frequently are the most isolated from infected periodontal pockets, including *Bacteroides forsythus*, *Actinobacillus actinomycetemcomitans*, *Campylobacter* spp., *Capnocytophoga* spp., *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Eikenella corrodens*, and *Prevotella intermedia*, there are also oral spirochetes and are thus recognized as potential periodontal pathogens (Socransky and Haffajee, 1992).

Porphyromonas gingivalis (*P. gingivalis*) is the second intensively studied probable periodontal pathogen and considered a major pathogen in chronic periodontitis (Haffajee and Socransky,1994). It produce a number of virulence factors and extracellular proteases, such as lipopolysaccharide, capsule, gingipain, fimbria and so on, resulting in the destruction of periodontal tissues (Hayashi *et al.*, 2012)

Nutritionally variant *streptococci* (NVS) are pleomorphic Gram variable bacteria showing fastidious growth requirements and is a common cause of infectious endocarditis in cases that are negative by blood culture. Four bacterial species have been identified as NVS: *Abiotrophia defectiva* , *Granulicatella adiacens* , *Granulicatella elegans* , and *Granulicatella balaenopterae*.(Hugo *et al.*, 2015). *G. adiacens* formerly described as a member of nutritionally variant *streptococci* (NVS) It is found to account for 85% of the NVS in the human mouth making it the most common type (Ohara-Nemoto *et al.*,1997). It colonizes the oral cavity, intestinal and genitourinary tracts as normal flora (Ruoff, 1991). This research aim to investigate the distribution of *G. adiacens* and *P. gingivalis* among periodontitis in gingivitis patient with orthodontic wire.

Key words: *G. adiacens* and *P. gingivalis*, orthodontic wire, PCR technique.

Materials and Methods

Sample collection: 78 samples of gingival swab have been collected from patient with orthodontic wires that suffering from gingivitis whom visited private dental clinics and 71 samples were collected from patient without orthodontic wires. All samples were collected from the mouth firstly by rolling a sterile cotton swab across the gingival region in lower and upper gum and by using dental floss for sample collection from sub-gingival region then swabs were cultured on MacConkey agar and Blood Agar base, incubated an anaerobically at 37°C for 24- 48 hr. for primary isolation of bacteria.

Molecular Bacterial Identification: PCR technique was used for molecular identification of *G. adiacens* using *16SrRNA* (Yat Woo *et al.*, 2003) and *P. gingivalis* using *IS1126* (Park *et al.*, 2004).

Extraction of DNA: Boiling method that described by Sambrook and Russell (2001) was carried out for DNA extraction. Briefly, an overnight of brain heart infusion culture (10 ml) of bacterial isolates were centrifuged at 6000 rpm/10 min and the pellet was washed twice with STE buffer and incubated with lysozyme for 10 min at room temperature, then heated to boiling for 5min and incubated in ice bath for 10 min. the mixture was centrifuge for 30 min at 15000 rpm. The supernatant was transferred to new Eppendorf tube and mixed with 0.7 v:v of isopropanol and incubated in ice bath overnight. DNA was recovered by centrifugation at 10000 rpm/10min and the pellet was washed with70% ethanol and preserved with 100µl of TE buffer (Tris-base and Na_2EDTA).

Amplification of target gene: monoplex PCR was used to amplified *16SrRNA* using LPW200F-GAGTTGCGAACGGGTGAG- and LPW200 R- CTTGTTACGACTTC ACC, and amplified *IS1126* using PI F-CCCGGCTTATGACGTGATTTCTCT, and PI R-CTGTTGCG TTTGTGCCCTTGTGC. PCR mixture with final volume of 20µl consist of 5µl of master mix (2.5U-iTag DNA polymerase,2.5mM dNTPs,1X reaction buffer and 1X Gel loading buffer), 3µl of each forward and revers and 6µl of DNA template. A condition of PCR thermocycler (Biometra, USA) involved 94°C for 2min followed by 30 cycle of 94°C for 2min, 55°C for 1min and 72°C for 2min with final

extension 72^{O}C for 10min. Multiplex PCR for amplification of input and output of *IS1126* using PI and PIRC(1-AGAGAAATCACGTCATAAGCCGGG- and 2-GCACAAGGGCACAAACGCAACAG). PCR mixture and condition was carried out as explained above. The resulted amplicon was electrophoresis on 1% agarose gel stained with 0.5µg/ml of ethidium bromide at 80V. for 1hr. and photographed. (Yat Woo *et al*., 2003; Park *et al* ., 2004)

Antibiotic sensitivity tests: The test was done for antibiotic resistance detection pattern of *G. adiacens* and *P. gingivalis* to six antibiotics belong to five different class of antibiotic as mentioned in table (1). Disk diffusion method was used as described by Kirby *et al*.(1969) Inhibition zone diameter was compared with CLSI (2010).

Table 1: Commercial Antibiotic Disk

Class	Subclass	Antibiotic	Symbol	Content
Beta-lactam/beta-lactamase inhibitor combinations	Non	Amoxicillin/Clavuanic acide	AMC	30ug
Cephems(parenteral)	Cephalosporin III	Cefotaxime	CTX	30ug
		Ceftazidim	CAZ	30ug
Macrolides	Non	Erythromycin	E	15 ug
Aminoglycosidase	Non	Amikacin	AK	10ug
Peptide	Non	Bacitracin	B	10ug

Mutation experiment: To evaluate the role of orthodontic wire as a mutagenic agent to bacterial isolates, two orthodontic wires have been chosen. Also four isolates of each *G. adiacens* and *P. gingivalis* has been carried out using a method described by Lentino *et al.*,1993 with some modification that include using crushing orthodontic wire in which 0.95 mg/10ml of stainless steel and 0.45mg/10ml of Nickel Titanium (NiTi) were added to BHI broth. The morphology of bacterial isolates treated with orthodontic wires as well as antibiotic resistance pattern were comparing with control after 24, 48, 72 and 96 hrs.

Statistical analysis

Least signifigant differences (LSD) and chi sequare (X^2) were used for analysis of our results.

Results and Discussion

Results that from culturing of 149 gingival specimen collected from patient with orthodontic wires of each gender: male (34 sample) and female (115 samples) their age group range from 17 onward showed that 54 isolates were belong to *G. adiacens* and 4 990-isolate belong to *P. gingivalis* while 91 isolates described as un identified bacteria.

The result of gel electrophoresis of amplicon resulted from amplification of *16S rRNA* of *G. adiacens* showed that 54 (36,2%) isolates were belong to *G.adiacens* by appearance of 1410 bp band on agarose gel stained with eithidium bromide as showed in figure (1)

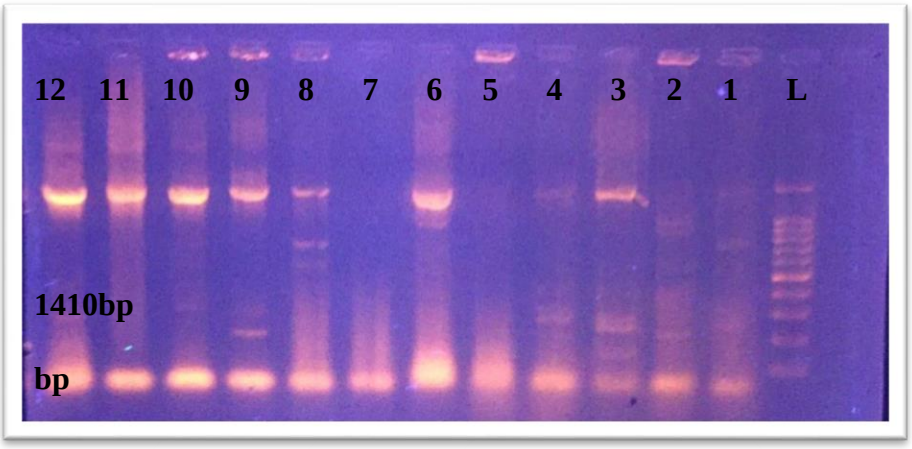


Figure (1): Gel electrophoresis of PCR product of *16S rRNA* amplicon of *Granulicatella adiacens* with 1410 bp. Lane (L) DNA marker (100bp), Lanes (3,6,8,9,10,11,12) positive result to *G. adiacens* (1% agarose, 80 Volt for 1hr)

While 4 (2,6%) isolates were belong to *P.gingivalis* by appearing of amplicon with molecular weight 690 bp on agarose gel stained with ethidium bromide as showed in figure(2). Multiplex PCR for amplification of IS1126-based PCR using PI1RC and PI2RC primers showed no amplicon has been appeared on agarose gel electrophoresis.

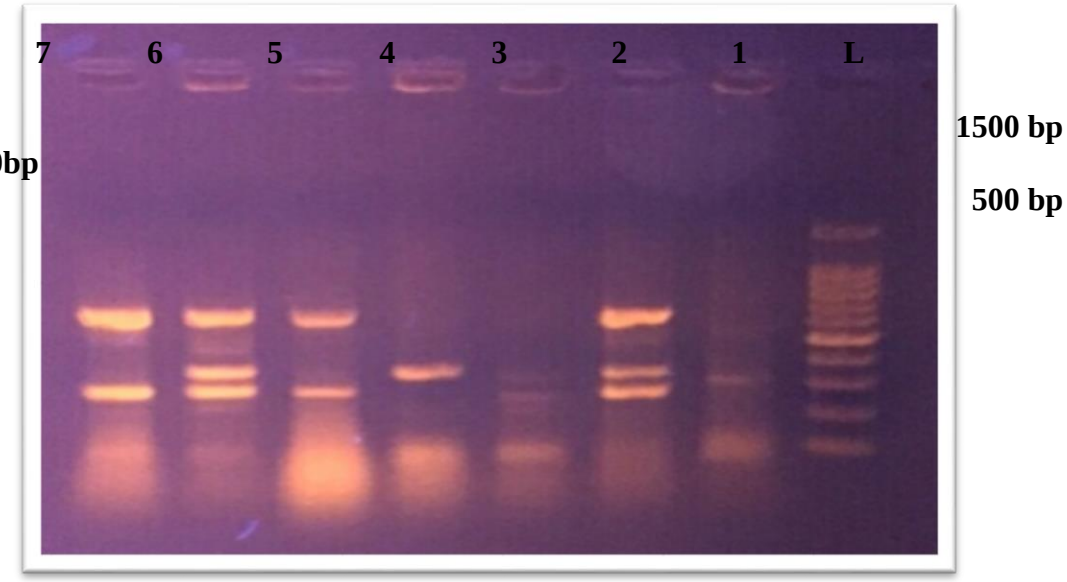


Figure (2): Gel electrophoresis of PCR product of *IS1126* (PI) of *Porphyromonas gingivalis* (amplicon with 690 bp). Lane (L) DNA marker (100-bp ladder), Lanes (2,5,6,7) positive result to *Porphyromonas gingivalis* (1gm agarose, 80 Volt for 1hr).

Molecular genetic methods have been widely used to investigate the bacterial diversity in various environments, including the human oral cavity microbiological diagnosis have not only been used to detect uncultivated bacteria only but also to identify cultivable bacterial species with superior specificity when compared with traditional culture-based methods (Song, 2005).

Most recent DNA studies have reported increased rates of detection of *G. adiacens* in periodontitis (Belstrom *et al.*, 2014), and so in endodontic infections (Hsiao *et al.*, 2012) Shimoyama *et al.*, (2011) used multiplex PCR as a rapid and highly sensitive identification method which is 16S rRNA PCR for *G. adiacens* by using primers set method for bacterial identification in roughly 4 h (Ohara-Nemoto *et al.*, 1997).

The genome of *P. gingivalis* have multiple copies of IS*1126*, a number strains of *P. gingivalis* were analysed by Southern blot analysis by using IS*1126* as a probe of therefor the value of this element has been used as a rapid epidemiological tool for identification of specific strain of *P. gingivalis* (Maley and Roberts, 1994).

Bactria that associated with periodontitis and gingivitis are not detectable when using standard culture techniques only and are extremely difficult to identify (Iwai **.** 2009). Another potential source of error in the culturing procedure for anaerobic bacteria belong to the processing of samples including transport media (Syed and Loesche,1972). Therefor polymerase chain reaction (PCR) method was used for DNA detection of oral bacteria (Toyofuku *et al.*, 2011)

The development of quantitative real-time PCR has enabled the sensitive and accurate determination of the cell number of individual species in subgingival plaque samples, (von Wintzingerode *et al.*, 1997). The efficiency of PCR assays in detecting microorganisms depends on collection of sample, PCR methodology, validation, and the interpretation of each PCR analysis (Fenollar *et al.*, 2006). Suggests that approximately 415 species are likely to be present when using PCR and sequence analysis 16S rRNA from bacteria in subgingival plaque (Paster *et al.*, 2001). The agreement between culture and PCR method in detecting the absence of *P.gingivalis*, when PCR was performed with the bacterial suspension obtained after cultivating of plaque samples supposed that there were no viable bacteria as well as PCR will detect not only viable but also moribund and dead cells (Van Assche *et al.*, 2009).

Detection limit could be explained the discrepancy between PCR-based and culture based studies for PCR the detection limit is typically 25–100 cells while for culturing 10^3–10^4 bacteria are required before detection the sensitivity of bacterial culturing is theraby low especially for non-selective media and therefore low numbers of a specific pathogen in a subgingival sample will remain undetected. (Van Assche *et al.*, 2009).

About one-half of the more than 700 different species of bacteria were detected in the humans oral cavity remain to be cultivated and are known only by using of 16S rRNA gene sequences (Kazor *et al.*, 2003). This approach has been used to detect uncultivated bacteria directly in samples from subjects with periodontal disease in an attempt to establish correlations with etiology of disease (Kumar *et al.*, 2003).

The result showed a high distribution of acute gingivitis in age group 17-27 among upper and lower gums compared with other type of gingivitis and other age group where their percentage were 32 and 28 in both lower and upper gum respectively as showed in Table (2) Also astatical analysis showed a significant differences between lower and upper gum in acute stage in age group (17-22) years in compartion with chronic at $p < 0.05$

Results showed widely distribution of bacterial isolates among chronic and acute gingivitis in which *G.adiacens* may represented a main causes of acute gingivitis in upper gum (18.5%) followed by *P.gingivalis* (0%). The same results were obtained in lower gum where *G.adiacens* incidence in a high percentage (11.1%). While in state of chronic gingivitis in upper gum *G.adiacens* was (11.1%) whereas in lower gum *G.adiacens* was (9.25%). While in state of non-orthodontic patient, *G.adiacens* appear with high dominance compared with *P.gingivalis* where the percentage of isolation were (20.3%) and (25%) respectively in upper gum while in lower gum *G. adiacens* was the most common as showed in Table (3). Also a statical analysis showed a significant to to *G.adiacens* in lower gum of non-orthodontic patient.

Granulicatellas occur relatively among other dental infections (Belstrom *et al*., 2014), it is known as nutritionally variant streptococci (NVS) due to their requirement for pyridoxal

or other additional agents to be incorporated into standard media for accurate laboratory isolation (Ruoff, 1991). Pyridoxal is required for coenzymatic transformation of L-alanine to D-alanine, which is necessary for production of peptidoglycan (Ruoff, 1991). Accurate identification of it can be difficult because of the pleomorphic nature and variable Gram-staining characteristics of the organism (Ruoff, 1991). limitation of nutrient can cause morphological pleomorphism may be as a result to bacterial growth unbalanced related to the limitation of nutrient (Frehel *et al*., 1988).

Table (2): Distribution of gingivitis types in ortho and non-orthodontic patient according to the age group with location of samples.

Age group	Orthodontic patient with gingivitis				Non orthodontic patient with gingivitis	Total
	Type of gingivitis					
	Lower gum		Upper gum			
	Acute	Chronic	Acute	Chronic		
17-22	32 (91.4%)	3 (100%)	28 (93.3%)	4 (100%)	71 (92.2%)	138 (92.6%)
23-28	3 (7.89%)	0 (0%)	3 (7.89%)	0 (0%)	3 (3.89%)	5 (3.35%)
29-35	3 (7.89%)	0 (0%)	2 (6.06%)	0 (0%)	3 (3.89%)	6 (4.02%)
Total	38 (25.5%)	3 (2.01%)	33 (22.1%)	4 (2.68%)	71 (47.6%)	149 (100%)
Calculated X^2	2.15		1.97			
Table X^2	0.71 P < 0.05					

Table (3) Distribution of bacterial isolates in ortho and non-orthodontic patient according to the type of gingivitis with location of samples.

Study group	Location	*G.adiacens*	*P.gingivalis*	Unidentified bacteria	Total
Non orthodontic	Upper	11 (20.3%)	1 (25%)	6 (21.4%)	33 (22.1%)
	lower	16 (29.6%)	3 (75%)	7 (25%)	38 (25.5%)
Orthodontic (Acute)	Upper	10 (18.5%)	0 (0%)	4 (14.2%)	30 (20.1%)
	Lower	6 (11.1%)	0 (0%)	3 (10.7%)	22 (14.7%)
Orthodontic (Chronic)	Upper	6 (11.1%)	0 (0%)	6 (21.4%)	16 (10.7%)
	Lower	5 (9.25%)	0 (0%)	2 (7.14%)	10 (6.71%)
Total		54 (36.2%)	4 (2.68%)	28 (18.7%)	149 (100%)
Calculated X^2		2.11			
Table X^2		0.71 P < 0.05			

During our study a correlation has been found between *S. aureus* and *G. adiacens* in which *G. adiacens* was occur around *S. aureus* colonies when culturing on blood agar base (unpublished data) this due to ability of *S. aureus* colonies to hemolyzed erythrocyte resulting high amount of pyridoxal an important substance that required for the growth of *G. adiacens* (Versalovic *et al*., 2011). While other studies

showed that *G. adiacens* unable to grow when culturing in trypticase soy agar with 5% of sheep blood because of their fastidious growth requirements (Frenkel & Hirsch, 1961). Many studies demonstrated that other organisms such as staphylococci, streptococci (excepting *Streptococcus pyogenes*), Enterobacteriaceae, and yeasts may support the growth of *G. adiacens* by supplement of pyridoxal or L-cysteine (Frenkel & Hirsch, 1961). Deficient forms of the cell wall developed as a result of antibiotics exposure so the appearance of Gram morphology appearance of cell-wall deficiency in *G. adiacens* remained the same after repeated sub culturing (Bottone *et al.*,1995).

P. gingivalis is considered the major pathogen among anaerobic Gram-negative bacteria that cause periodontitis (Nishihara and Koseki, 2004). Which produce porphyrin pigments when grow on blood agar (dark brown/black pigments) (Bachrach *et.al.*, 2011) Our results showed that *P. gingivalis* have the ability to produce black colonies when grow on blood agar base due to aggregation of hemin on its cell wall as a result from using iron transport system (Ogrendik *et al.*, 2005). This properties represented an important feature that recognized an opportunistic isolates from un virulent isolates when grow on heme-limited medium (McKee *et al.*, 1986)

P. gingivalis does not produce siderophores to sequester and transport iron but its gingipains mediate the uptake of iron from hemoglobin, heme proteins, and ferritin unlike other gram-negative (Sroka *et al.*, 2001). The source of metabolic energy of *P. gingivalis* obtained by fermenting amino acids and this property is very important which enable it to survive in deep periodontal pockets where sugars are extremely scarce (Kolenbrander *et al.*, 2011). Is found in close proximity and interacts with the juxtaposing gingival tissue when considering its location in multispecies subgingival biofilm communities therefor it represent as a late colonizer (Zijnge *et al.*, 2011). The ability of *P. gingivalis* to colonized subgingival plaque may due to their ability to tolerance anaerobic condition and neutral pH (Takahashi and Schachtele. 1990)

Variations in *P. gingivalis* virulence occur because of the phenotypic expression which induced by both host and environmental factors while the variation within the same strain could also be due of recombinations and genetic rearrangements (Holt *et.al.*, 1999). Previous studies have tried to characterize more virulent types of *P. gingivalis* by the expression of various genetic rearrangements virulence factors e.g. biochemical activity, colony morphotypes, production of enzyme, antibiotic susceptibility, fimbriae, capsule formation antigenic properties, and their ability of adherence to various host cells (epithelial cells, neutrophils, hemagglutination, fibroblasts) (Holt *et al.*, 1999).

G. adiacens was detected in high level especially in the oral cavity of adult (Sato *et al.*, 1999; Aas *et al.*, 2005). It is associated with up to 2.3% of *streptococcal* bacteremia and up to 5% of *streptococcal* endocarditis and it was found more common than *A. defective* and much more common than *G. elegans* as an etiologic agent of bacterial endocarditis and in some cases of infectious crystalline keratopathies and corneal ulcers following penetrating keratoplasty and it was suggested that Co-infection with *S. aureus* or other *streptococcal* species may contribute to the growth of *G. adiacens* in vivo (Christensen and Facklam, 2001). It form important part of biofilm in dental plaque due to their ability to co-aggregate and grow of *G. elegans* and *A. actinomycetemcomitans* with *F. nucleatum* to form th "bridge organism". *Granulicatella* spp. have benefits for such this partnership for example, if *Granulicatella* spp. lack β-lactamase similar to some *streptococci in* mouth. (Kuriyama *et al.*, 2002).

P. gingivalis was detected in patients with periodontitis and in healthy subjects (Frandsen *et al.*,2001). Lamont *et al.*, (2013) identified *P. gingivalis* (Pg) as bacteria that form biofilm and cause gingivitis and

periodontitis. *P. gingivalis* present in high level in advanced forms of periodontitis and play important role in the pathogenesis of it (Scher *et al.*, 2012; Abusleme *et al.*, 2013). It was detected in deep periodontal pockets of adults (van Winkelhoff *et al.*, 2002), and correlated with periodontal pocket depth (Grossi *et al.*, 1995). As well as there were low numbers of *P. gingivalis* is present when found in healthy cases. (Marsh, 2003). Riep *et al.* (2009) reported that *P. gingivalis* could also be frequently isolated from healthy controls this in contrast to other studies where Haffajee and Socransky 1994 showed that *P. gingivalis* is uncommon or found in low numbers in healthy individuals and those with gingivitis, while it is more frequently detected in those with more destructive forms of disease. Samples from 3.2% of children and adolescents without periodontitis showed a positive reaction to *P. gingivalis*-specific primers (Tamura *et al.*, 2005), studies reported a high correlation between rate of detection of *P. gingivalis* and the age (Ooshima *et al.*, 2003). while another study noted that *P. gingivalis* may be difficult to transmit or require a longer period of time for colonization (Umeda *et al.*, 2004). *P. gingivalis* has been detected at a high level (50.25– 89.4 %) in periodontitis patients but also at a low level (23.1–36.8 %) in healthy individuals (Missailidis *et al.*, 2004; Zhao *et al.*, 2007). Also *P. gingivalis* was detected in 77.3% of samples from early periodontal patient using culture method (Kamma *et al.*, 2004). There is a strong evidence for a significant association between rheumatoid arthritis and periodontitis. *P. gingivalis* which is the major etiologic factor in periodontitis and gingivitis facilitates the development and progression of collagen induced arthritis (Adamowicz *et al.*, 2014).

Mutation experiment

To explain the role of orthodontic wire on bacteria isolates a mutation experiment has been carried out. Four isolates of each *G. adiacens*, and *P.gingivalis* were randomly selected.

Results of 24 hr of incubation of *G. adiacens* in BHI broth containing stainless steel and NiTi wire showed no change in the colonies appearance comparing with control group (figure3A), While a greenish discoloration of the colonies was obtained after 48 h of incubation of *G. adiacens* with NiTi wire (figure 3B). Wheres after 72 hr of incubation no discoloration of the colonies was observed (figure3C). Finally, after 96 hr of incubation of *G.adiacens* in both of NiTi and stainless steel wire containing broth appear the same of control when culturing on BHI agar (figure 3D).

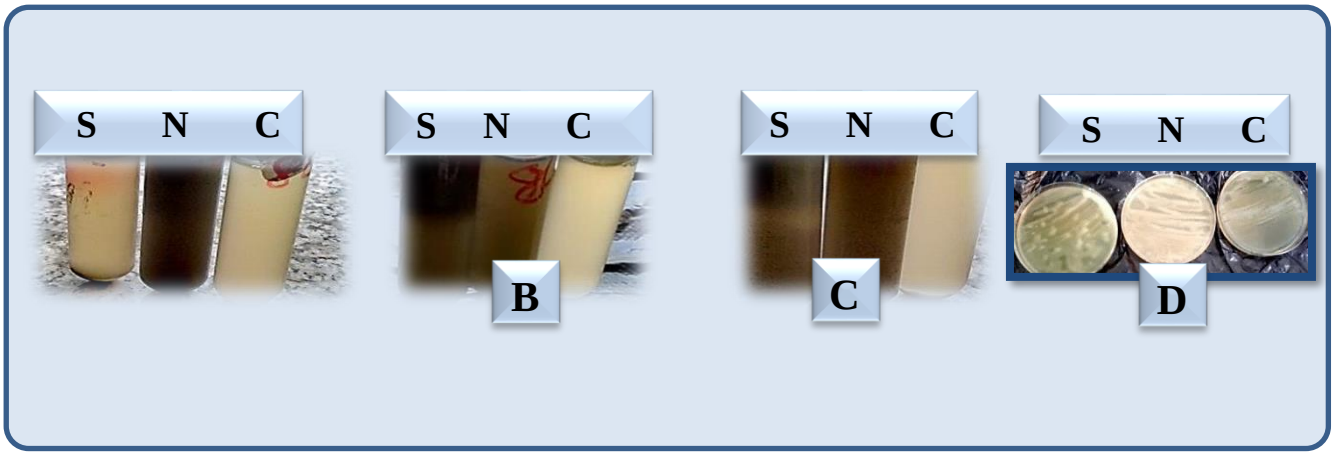
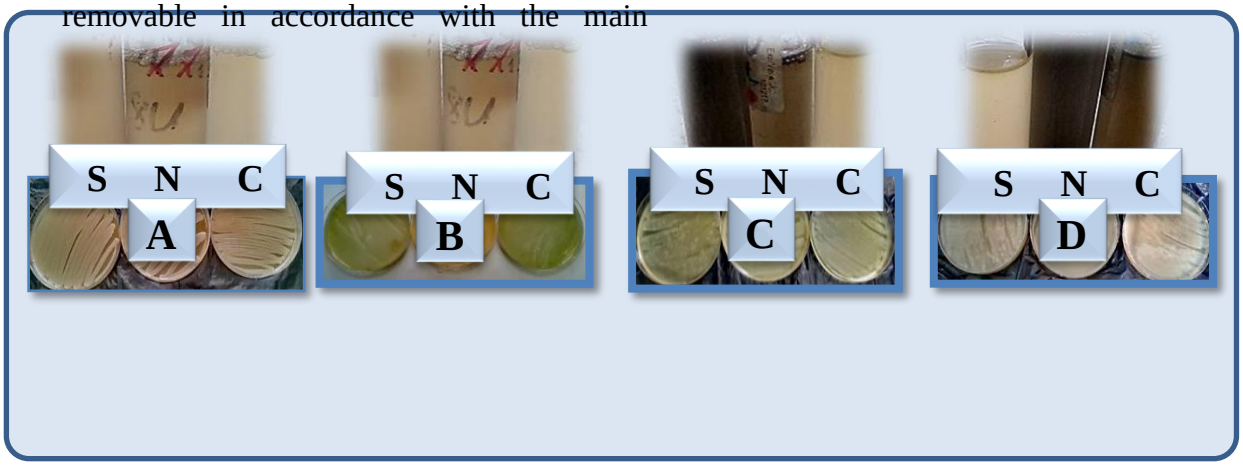


Figure (3): Morphological characteristic of treated *Granulicatella adiacens* grow on brain heart infusion agar. **A**- 24 hours incubation period; **B**- 48 hours incubation period **C**-72 hours incubation period **D**-96 hours of incubation period. N= treated with Nikle-titanium wire, S= treated with stainless steel wire, C=control.

On the other hand the result of 24 hr of incubation of *P. gingivalis* on BHI broth containing NiTi and stainless steel wire showed no change in the appearance of colonies when culturing on BHI agar in comparing with control (figure 4A), while the result of 48 hr of incubation of *P. gingivalis* cause greenish discoloration of colonies of control and stainless steel wire containing broth after culturing on BHT agar (figure 4B). After 72 and 96 hr no change in the color of the colonies has been occurred as shown in figure (4 C and D) respectively.

Many appliances are available either fixed or removable in accordance with the main purpose of the treatment (Chung and Font, 2004). Orthodontic fixed appliance therapy is the commonest mode of treatment and the most commonly used orthodontic materials are brackets, tubes, band material, ligating materials and arch-wires. These materials facilitate the microbial adhesion and greatly inhibit oral hygiene and provide new retentive areas for plaque and debris which in turn predisposes the wearer to increased microbial burden and possibility of subsequent infection (Magno *et al*., 2008).



Figure(4): morphological characteristic of treated *Porphyromonas* gingivalis grow on brain heart infusion agar A- 24 hr of incubation period B-48 hr of incubation period C-72 hr of incubation period D-96 hr of incubation period. N= treated with Nikle-titanium wire, S= treated with stainless steel wire, C=control.

The result of this study indicate an increasing in the level of pathogenic bacteria when comparing healthy and gingival case (Table 3). While *P. gingivalis* obtained from healthy only this due to mistaken in collection of samples because we discarded the suspected colonies during our work and a mistake in primary diagnosis of *P. gingivalis* which lead to loss of bacteria during cultivation. Multiplication of decaying bacteria increased significantly in the presence of fixed appliances in the mouth for one to two years (Chany *et al.*, 1999).

Percentage of *P. gingivalis* increased significantly after wearing orthodontic appliance and the increase of it was significantly related with the development of gingivitis in orthodontic treatment (Huang and Xiao. 2010; Wang *et al.*, 2011). Peros, *et al.* (2011) reported new data on the duration of salivary microbial changes induced by the placement of fixed orthodontic appliances they noted the success of antimicrobial preventive measures for orthodontic patients with proper timing, Such measures should be applied between sixth and twelfth weeks of orthodontic therapy which is the time where *St. mutans* and *Lactobacillus* spp. increase in the saliva in which their increase significantly in 6 months after the insertion of fixed orthodontic appliances. According to Topaloglu-Ak, *et al.* (2011) the negative effect of microbial flora can occur at long-term utilization of appliances of orthodontic and so increase the risk of carious lesions.

Exposure of bacterial isolates to NiTi wire results in changing the color of culture media this may due to the fact that NiTi alloys compose of 55% nickel and 45% titanium (Roach, 2007) which lead to effect on chemical properties of media as well as the metabolic activities of bacterial isolates. NiTi archwires were considered better than stainless steel alloys due to their elasticity of 20% higher than stainless steel alloys (Chaturvedi, 2010), but also has a disadvantage which include a decrease in mechanical properties due to corrosion processes (Cai *et al.*, 2010). NiTi archwires were covered with Teflon based materials, composite resins, hydrogenated carbon or zirconium dioxide, which restricted corrosion and restrict the release of Ni by 80% without alter the mechanical properties of the archwires (Ohgoe *et al.*, 2007; Elayyan *et al.*, 2008), this phenomena may also play role in altering the color of culture media. Clinical oral manifestations in orthodontic patients such as gingival hyperplasia and periodontitis might be associated with an inflammatory response elicited by the corrosion of orthodontic appliances and then subsequent release of nickel. (Genelhu *et al.*, 2005.) Eliades *et al.* (2000) reported alteration in the composition of surface NiTi archwires after intra-oral exposure for 1–6 months due to the occurrence of amorphous precipitates and microcrystalline particles in proteinaceous biofilm.

Stainless steel arch wires have been used as orthodontic wires with a wide range of applications in both the fixed and removable appliances (Brantley *et al.*, 2002). Studies on it showed that the smoothness of their surface is responsible for the decrease in count of *Streptoccocus* colony on it where the adhesion ability in the coated and non-coated group was increased by the extended incubation time and was the highest after three hours of incubation (Yu *et al.*, 2011). So the extended incubation time increased the adhesion of cariogenic *Mutans streptococci* (Amini *et.al.*, and D'Anto` *et al.*, 2012). The action of microbial colonization is twofold either take up and metabolize metals from alloys or microbial byproducts with the metabolic processes may alter the conditions of the microenvironment (ie, decreasing the pH and therfore contributing to the initiation of the corrosion process) (Palaghias,1985).

Aerobic, facultative and anaerobic bacteria favouring the corrosion process Aerobic bacteria utilize the simple sugar then enter into glycolysis and TCA cycle releasing carbon dioxide (Gerhard, 1985) The facultative bacteria enter into the fermentative pathway utilizing the simple sugars and produce organic alcohols, acids and CO2, Organic acids formation cause reduction of pH thereby it favoring corrosion. facultative in the anaerobic zone utilize the lactate as carbon source and reduce sulphate to sulphide then sulphide combines with iron to form ferrous sulphide**.** The sulphide produced by sulfide reducing bacteria (SRB) enters into the interface of the anaerobic and facultative zones where it gets oxidized by sulphate oxidizing bacteria to sulphate, sulphuric acid is also formed which cause reduction of the pH and cause tooth decalcification and corrosion of metallic implants because of its corrosive nature. Low pH provide favorable environment for aerobic microbes such as iron oxidizing bacteria (Maruthamuthu *et.al.*, 2005) MnO_2, FeO, Fe_2O_3 These metal ions combine with the bacterial end-products along with the chloride ion in the electrolyte of saliva to form more corrosive products like ferric chloride ($FeCl_3$), manganese chloride ($MnCl_2$), etc. This leads leaching of metal with subsequent release of chromium and nickel into the body and then decalcification of teeth (Christopher *et al.*, 2004).

Antibiotic sensitivity test

Antibiotic resistance patteren was detected for both origin isolates and mutated isolates to explain the effect of orthodontic wire on increasing or decreasing of antibiotic resistance manner of isolates.

The result of antibiotic resistance pattern after 24hr incubation of origin isolates of *G.adiacens* showed a variation in antibiotic resistance pattern to tested antibiotics

(table-4), while after exposure of these isolates to NiTi and stainless steel wire a variation in antibiotic resistance among same isolate was observed in which isolate that sensitive to some antibiotics became resistance to it and visversa as shown in table (4). An increased in antibiotic resistance pattern was observed after 48,72 and 96 hr. of incubation with each wires.

Also, the same results were obtained when incubation of *P. gingivalis* with each wires in comparison with original isolates also after 24, 48, 72 and 96 hr. of incubation (Table5).

Table (4). Antibiotic resistance pattern of *Granulicatella adiacens* (origin and mutated isolates) to certain antibiotic after different incubation period

Incubation Period	cefotaxime			Bacitracin			ceftazidim			Augmentn			erthromycin			amikacin		
	C	**N**	**$**	**C**	**N**	**$**	**C**	**N**	**$**	**C**	**N**	**$**	**C**	**N**	**$**	**C**	**N**	**$**
24hr	**27.8**	**29.3**	**24**	**5.8**	**3.5**	**9.3**	**14.3**	**12.8**	**10.8**	**12.8**	**5.8**	**11.8**	**12.3**	**6.5**	**6.8**	**19**	**21.8**	**22.3**
48hr	**24.8**	**28.8**	**14.8**	**0**	**3.3**	**2.3**	**13.5**	**16.3**	**5.5**	**3**	**5**	**6.3**	**2.5**	**2.5**	**5.5**	**16.8**	**17.5**	**18**
72hr	**17**	**29.8**	**25.5**	**10**	**0**	**0**	**5**	**14**	**12.3**	**5.8**	**3.3**	**4**	**4.5**	**0**	**2.3**	**16.8**	**14.3**	**15.3**
96hr	**21**	**23**	**25.5**	**0**	**0**	**2.3**	**7**	**18**	**15**	**0**	**0**	**0**	**0**	**0**	**5**	**17.3**	**21.3**	**20.5**
LSD	6.7Sign $ 48 hr			3.3 Sign N, $ 72 hr			3.5Sign $ 48 hr			5.5 Sign N, $ 96 hr			2.2 Sign N 72 , 96 hr			1.8 Sign N 72hr		

N=Nikle-titanium wire, $=stainless steel wire, C=control, R=Resistant, S= senstive

Table(5). Antibiotic resistance pattern of *Porphyromonas* gingivalis (origin and mutated isolates) to certain antibiotic after different incubation period

Incubation Period	cefotaxime			Bacitracin			ceftazidim			Augmentn			erthromycin			amikacin		
	C	**N**	**$**	**C**	**N**	**$**	**C**	**N**	**$**	**C**	**N**	**$**	**C**	**N**	**$**	**C**	**N**	**$**
24hr	**36.5**	**22.8**	**26.5**	**9.5**	**4**	**10.3**	**14.3**	**12.5**	**10.5**	**15**	**11**	**14**	**20.3**	**13.5**	**15.5**	**16.3**	**22.3**	**27.8**
48hr	**28.8**	**31.3**	**36**	**1.8**	**0**	**0**	**13**	**13**	**14.8**	**0**	**0**	**2**	**4.5**	**0**	**2.5**	**16.5**	**16.5**	**16.3**
72hr	**27.5**	**18.5**	**26.8**	**0**	**0**	**2**	**15.8**	**8.8**	**14**	**2**	**2.5**	**2.8**	**2**	**3.3**	**5.3**	**15.3**	**15.3**	**15**
96hr	**15.5**	**16**	**20.5**	**0**	**0**	**2.5**	**0**	**10**	**12**	**0**	**0**	**0**	**0**	**0**	**0**	**17.5**	**17.3**	**18.5**
LSD	2.4 Sign $ 48 hr			1.3 Sign $ 24 hr			1.1 Sign $ 48 hr			3.2 Sign N, $ 48 , 96 hr			5.6 Sign N, $ 96 hr			4.4 Sign $ 72hr		

N=Nikle-titanium wire, $=stainless steel wire, C=control, R=Resistant, S= senstive

Antimicrobial drug susceptibility patterns, In vitro do not correlate well with clinical response to treatment and there is poorly respond to antimicrobial treatment from NVS infections with significant rates of microbiological failure and relapse rates after treatment have been showed for NVS infections than with *streptococci* and related genera (Adam *et al.*, 2015). *G. adiacens* has been recorded to be resistant to penicillin, and resistance to extended-spectrum cephalosporins and newer fluoroquinolones (Tuohy *et al.*, 2000).in contrast to Ruoff. (1991) that showed that NVS were moderately susceptible to penicillins, clindamycin, chloramphenicol, erythromycin, rifampin, and vancomycin and variably susceptible to cephalosporins . The emergence of macrolide resistanc of *G. adiacens* that cause endocarditis is associated with high mortality (Bouvet and Acar, 1984). Woo *et al.*, 2003 reported that three out of nine isolates of NVS were resistant to erythromycin, clarithromycin and azithromycin while Cargill *et al.*, (2012) reported that isolates was susceptible to clindamycin, rifampin, and vancomycin, and it was resistant to penicillin, cefotaxime, ceftriaxone, and meropenem.

positive responses have been reported with amoxicillin/clavulanic acid in the periodontitis treatment (Van Winkelhoff *et al.*, 2005). The fact that 12 % of the bacteria were resistant or intermediate resistant to amoxicillin but 100 % were sensitive to amoxicillin/clavulanic acid indicates resistance of *P.gingivalis* to β-lactam antibiotics which due to β-lactamase production (Blandino *et al.*, 2007).

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