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Molecular Identification of *Fusobacterium* Isolates and limitation of Biofilm Formation Adhesion Gene (*fadA*) in Dental Outpatients

Aws Ibrahim Sulaiman

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Abstract:

Fusobacterium are compulsory anaerobic gram-negative bacteria, long thin with pointed ends, it causes several illnesses to humans like pocket lesion gingivitis and periodontal disease; therefore our study is constructed on molecular identification and detection of the *fadA* gene which is responsible for bacterial biofilm formation. In this study, 10.2% *Fusobacterium* spp. were isolated from pocket lesion gingivitis. The isolates underwent identification depending on several tests under anaerobic conditions and biochemical reactions. All isolates were sensitive to Imipenem (IPM_{10}) 42.7mm/disk, Ciprofloxacin (CIP_{10}) 27.2mm/disk and Erythromycin (E_{15}) 25mm/disk, respectively. 100% of *Fusobacterium* spp. isolates had 16S rDNA gene (360bp.), whereas two isolates had *fadA* gene (232bp.)

Key words: Biofilm formation, *FadA*, *Fusobacterium* spp., 16S rDNA

Introduction:

The genus *Fusobacterium* includes several species of obligatory anaerobic, opportunistic pathogens from bacteroidaceae family, that bare a strong resemblance to certain *Bacteroides* species, under the microscope, they are long, thin, and tapered rods (normally spindle-shaped cells) with sharp ends, non-spore-forming, motile or non-motile, slender, spindle shape, fusiform or pleomorphic parallel rods, and show negative behavior to gram stain (1).

They are found in mucous membranes of humans and animals and infected epithelial cells of mouth and intestine and play an important role in early buccal cavity biofilm development and also in plaque biofilm colonization by co-aggregating with other pathogenic bacteria in buccal cavity, the best isolation of *Fusobacterium* spp. is from saliva or salivary fluid after concentration or from pocket lesion found between gingiva and surface tooth, the members of this microbe are resistant to vancomycin and susceptible to kanamycin and colistin. (2, 3).

They play an important role in gingivitis, osteomyelitis, periodontitis, appendicitis, and other invasive infections of many human organs like head, neck, lung, liver, heart and brain and can causes pregnancy complications (preterm birth, stillbirth and neonatal sepsis) because they can pass through umbilical cord (4, 5, 6).

Department of Biology, College of Science, University of Mosul, Mosul, Iraq.
E-mail: aws_isulaiman@yahoo.com

Fusobacterium spp. are the most abundant species found in buccal cavity, in ill and healthy humans and cause severe damage in different forms of periodontal lesions ,between mild reversible gingivitis form irreversible periodontitis form, they are viewed like islands in the mouth because of a special habitat in the human body characterized by liquid water near-constant presence, briefly the extreme temperature fluctuation, by an externally exposed hard surface (teeth) and by wide variation in carbon and nitrogen input, including a basal component (saliva is a complex mixture with limited sources energy for bacteria, the *fadA* attachment gene was identified to be involved in oral *Fusobacterium* spp. and highly conservative for them (7).

The aim of this work is to detect the biofilm formation adhesion gene in *Fusobacterium* isolates.

Materials and Methods:

Sample collection:

In this study thirty-nine swabs of bacteria were collected from patients (male: 23 and Female: 16) attending Special Dental Center/Al-Noor in Mosul city/ IRAQ suffering from pocket lesion gingivitis after being diagnosed by dentist and swabs were taken from the base of the periodontal pocket.

Samples were transferred to the scientific laboratory in biology Department, College of Science, University of Mosul, immediately after

collection by Fusobacterium Selective Medium (FSM).

Isolation and Identification of Bacterial Strains:

Swabs from pocket lesion gingivitis and the teeth surface were inoculated on Fusobacterium selective medium FSM (15g agar, 10g peptone, 5g Na_2HPO_4, 5g glucose, 3g beef extract, 2g soluble starch, 1g $NaNO_3$, 1g yeast extract, 0.5g L-cysteine·HCl.H_2O, 0.004g/1ml ethyl violet solution) according to Atlas and Snyder(8) with modification using vancomycin solution (0.1mg/1 ml) instead of bacitracin incubated at 37 °C for 24 hrs.; except Ethyl Violet and vancomycin solutions, all components dissolved in DDW to bring the volume 980.0ml after autoclaving aseptically, sterile Ethyl Violet solution and vancomycin solution 20 ml. were added to make one liter.

Furthermore, biochemical assays were carried out to confirm the identification of isolates included Catalase, Esculin hydrolysis, Urease, Carbohydrate fermentation (Glucose, Fructose, Lactose and Mannose) and Gram stain for detection of the fusiform shape with tapped ends and pleomorphic shape with rounded ends all bacterial isolates were incubated in anaerobic chamber for three days at 37 °C (1).

Antibiotics Susceptibility assay:

Ampicillin (AM_{10}), Ciprofloxacin (CIP_{10}), Gentamicin (CN_{10}), Erythromycin (E_5), Imipenem (IPM_{10}), Cepalothine (KF_{30}) Penicillin (P_{10}) and Trimethoprim (TMP_5) were used to determine the susceptibility of *Fusobacterium* isolates to antibacterial agent (9).

DNA Extraction:

The DNA was extracted according to Junior *et al.* (10) with little modifications. Briefly *Fusobacterium* isolates were inoculated in brain heart infusion broth distributed in 1 ml Eppendorf microfuge tubes for 24 h at 37 °C under anaerobic conditions. centrifuged at 14,000 rpm for 5 min and supernatants were discarded and the pellets re-suspended in 200 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA) and boiled for 15 min, then put in cooling ice-bath for 15 minutes subsequently centrifuged (5 min/14,000 rpm) at 25°C. The DNA concentration absorbance was at 260 and 280 nm (50.0 ng/ µl). Then DNA samples were stored at -20 °C until use.

Detection of 16S rDNA and *fadA* gene in *Fusobacterium* isolates:

PCR was conducted using GoTaq® G2 Green Master Mix, Promega Corporation-USA (Each PCR mixture consists of GoTaq® Green Master Mix 10 µl, forward primer 1 µl, reverse primer 1 µl, DNA template 4 µl, and Nuclease-Free Water 4 µl). The Primers were purchased from Alpha DNA Company (Montreal, Quebec-Canada) The primers for *Fusobacterium nucleatum* of 16S rDNA were forward (5-AGA GTT TGA TCC TGG CTC AG -3) and reverse (5-GTC ATC GTG CAC ACA GAA TTG CTG-3) to amplify 360-bp region of the 16S rDNA gene was achieved by program consist pre denaturation at 94 °C for 5 min. subsequently 30 cycles, each one of these cycles consisting denaturation 94°C for 30 sec annealing for 58°C 30 sec and extension 72°C for 1 min after that 10 min for final extension at 72°C. *fadA* primers forward 5-CAC AAG CTG ACG CTG CTA GA-3, reveres 5-TTA CCA GCT CTT AAA GCT TG-3 to amplify a 232-bp region of this gene.

While the polymerase chain reaction of *fadA* was achieved by pre denaturation at 94°C for 4 min for 30 cycles which included denaturation 94°C for 30 sec, 55.8°C for 30 sec and 72°C for 40 sec subsequently final extension at 72°C for 6 min. The amplicons were electrophoresed on 2% agarose gel in TBE buffer then visualized by staining with ethidium bromide dye and UV Tran-illumination device (3).

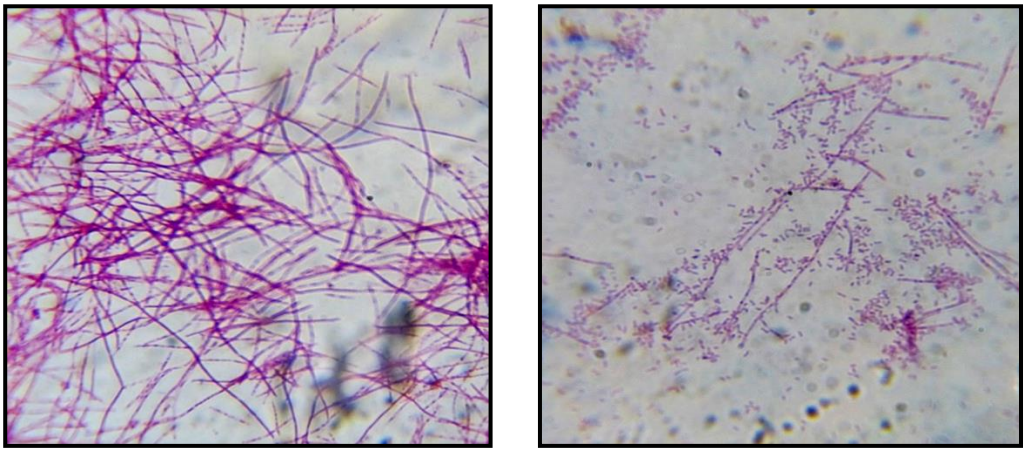
Results and Discussion:

From this study, 4 (10.2%) of *Fusobacterium* isolates were detected. this result somewhat was similar to the results of Hamad and Jasim, they obtained 12 % of clinical bacterial isolates from dental diseases belonging to *Fusobacterium* spp. and also our results are in agreement with some researchers and their study on microorganisms in dental plaque and the important role of dental caries and damage of tooth structure. (11)

The biochemical properties of *Fusobacterium* isolates were not able to produce catalase, not all were able to utilize glucose, fructose, and mannose. While the isolates varied in fermenting lactose, they produced urease and esculin hydrolysis (Table 1) and Figs. 1 and 2.

Table 1. Biochemical properties of *Fusobacterium* spp. isolates

Numbers of isolates	Gram stain	Catalase	Glucose	Fructose	Mannose	Lactose	Esculin hydrolysis	Urease
1	G- spindle ends long fusiform bacteria	-	-	-	-	-/+	-	+
9	G- pleomorphic shape with rounded ends	-	-	-	-	-	-	-
17	G- coccobacilli shape with rounded ends	-	-	-	-	-	-	+
18	G- spindle ends long fusiform bacteria	-	-	-	-	-	-	-



A **B**

Figure 1. *Fusobacterium* isolates under microscope 100X, A. fusiform shaped with tapered ends, B. pleomorphic shaped with rounded ends.

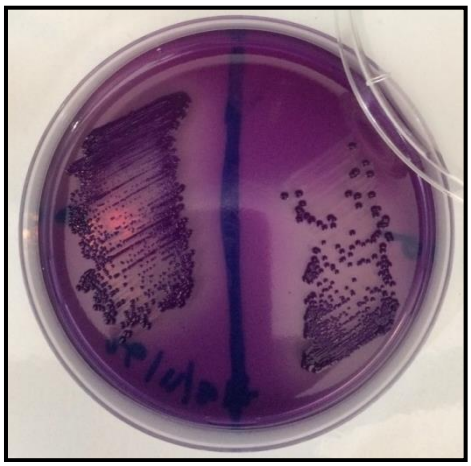


Figure 2. *Fusobacterium* isolate on Fusobacterium Selective Medium (FSM) incubated anaerobically at 37°C for 72 hrs. Left small colonies and right large colonies

The genus *Fusobacterium* exhibit more partnership than any other genus, because they congregate with early and last colonization of the habitat and have the most bridge microorganisms in the succession of species causing natural development of dental plaque, the best for transfer of genomic material happening by biofilms because they provide an optimum environment for exchange between cells and the DNA was trapped within the extracellular matrix (12).

Jensen and other workers (13) reported that *Fusobacterium necrophorum subsp. funduliforme* is part of the microbiota of human tonsils and in this study it was recorded that the *Fusobacterium* spp. in oral and buccal cavity also these results are in agreement with the result of Doron and *et al.* (14) when they found that *Fusobacterium nucleatum* is an oral anaerobe associated with periodontal disease.

All isolates in this study were sensitive to IPM_{10} (42.7mm/disk), CIP_{10} (27.2mm/disk) and E_{15} (25mm/disk) while these isolates were resistant to other antibiotics used in this study, the non-aerobic bacterial species possess several resistance mechanisms against beta-lactam antibiotics, including the production of beta-lactamase enzymes

(BLAs) *Fusobacterium* spp. one of them are found to be more resistant to antibiotics, like AM10 and P10 but our results differ with Abdulkareem study, because he found that most *Fusobacterium* spp. were resistant to AM10, P10 and E5 (15, 16).

The antibiotic erythromycin has the ability and effectiveness against various bacterial species, especially anaerobic ones, but in some cases, because of the acquisition of bacteria, the resistance traits become less effective especially *Fusobacterium* spp. (16)

These differences between this study and the other studies are due to the difference of bacterial samples; this will make the difference in percentage or isolates containing resistant genes for these antibiotics unlike us according to horizontal gene transfer (15).

Legaria and their colleague (17) found *Fusobacterium* spp. in their study sensitive to CIP_{10} and it has been found that the microbes are sensitive to this antibiotic, the determinants for antimicrobial resistance can be plasmid or chromosomally mediated, the purity of DNA in all isolates under study were 1.839 and concentration 50.0 ng/ µl at 260/280 nm.

16S rDNA gene with 360 bp was found in two isolates of *Fusobacterium* spp., which had different phenotypes. They may *Fusobacterium nucleatum* and *Fusobacterium necrophorum* these results somewhat agreement to Liu, *et al.* (18) Fig. 3.

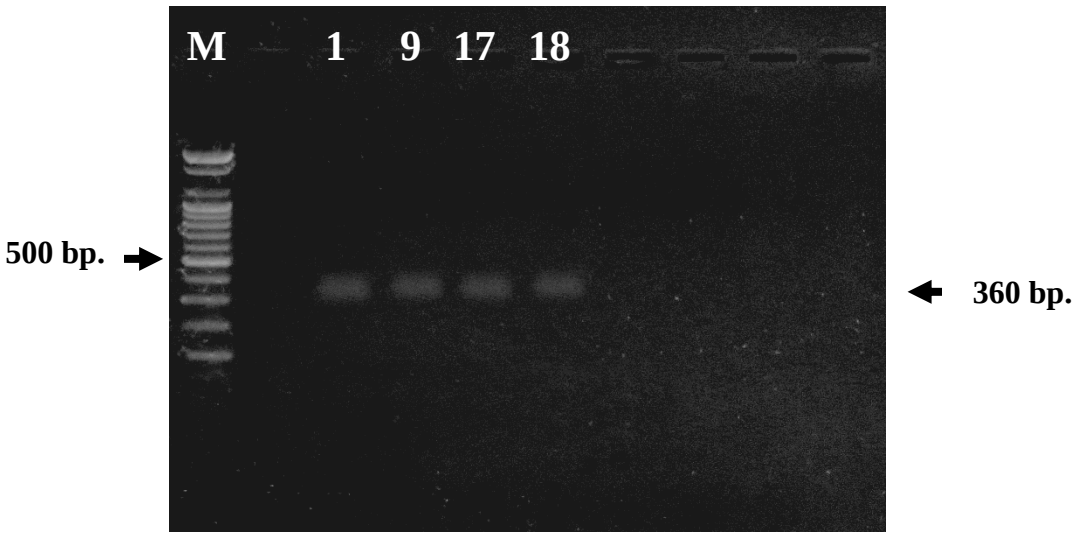


Figure 3.16s rDNA of *Fusobacterium* isolates (360bp.)

The results above disagree with a study done by Liu, and his coworkers and others (13, 18) which may be due to genetic, geographical and environmental changes. Fig. 4

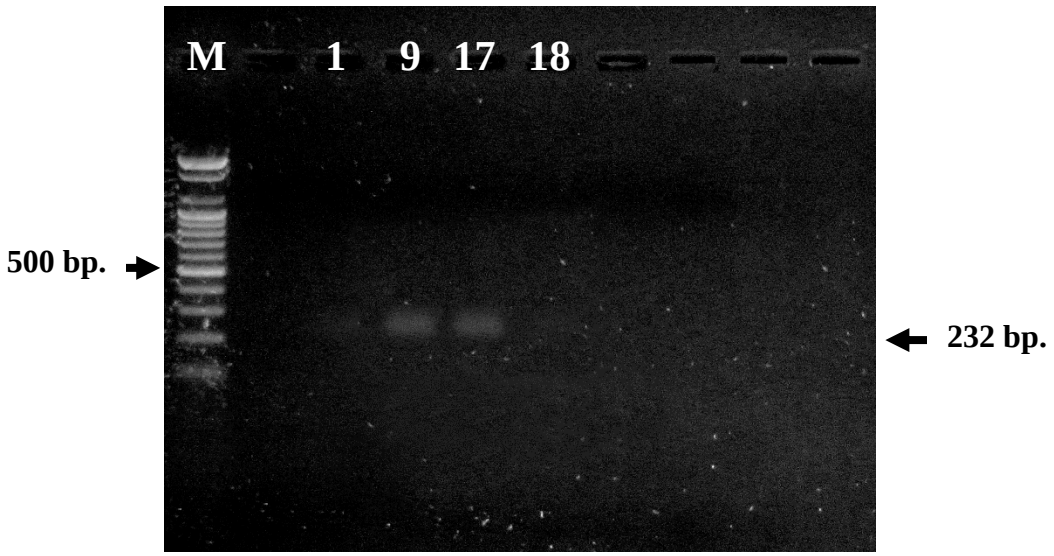


Figure 4. *fadA* gene (232 bp.) of *Fusobacterium* isolates

Conclusion:

An amplification of 16S rDNA is an efficient tool for rapid identification of *Fusobacterium nucleatum* and *Fusobacterium necrophorum* in clinical samples. In addition, Biofilm formation adhesion gene (*fadA*) was detected in 50% of the investigated *Fusobacterium* spp. this is an important risks which effect gingivitis lesions and inflammation. Patients must receive oral hygiene and advanced prophylactic treatment to maintain health of gingiva. We need more research to verify microorganism causing mouth diseases in human and advanced research for *Fusobacterium* spp. effect on buccal cavity hygiene for controlling pathogenic anaerobic bacteria.

Conflicts of Interest: None.

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التشخيص الجزيئي لـ *Fusobacterium* spp. والجين المسؤول عن تكوين الاغشية الحيوية عند مرضى الاسنان

أوس إبراهيم سليمان

قسم علوم الحياة، كلية العلوم، جامعة الموصل، الموصل، العراق.

الخلاصة:

ان الافراد التابعة لجنس Fusobacterium متشابهة الى حد كبير للأفراد التابعة لجنس Bacteroides من ناحية كونها سالبة لصبغة كرام ولاهوائية اجبارية، لكن افرادها يمتازون بأن خلاياهم طويلة نحيفة ذات نهايات مستدقة وبعضهم تمتاز بكون نهاياتهم دائرية وبالمجمل تشبه المغزل، تسبب عدة امراض للإنسان مثل التهابات جيوب اللثة وحول الاسنان لهذا هدفت الدراسة الى تشخيص هذه البكتريا جزيئيا وتحديد الجين المسؤول في تكوين الاغشية الحيوية اذ تم الحصول على (10.2%) من بكتريا *Fusobacterium* spp. من مجموع 39(89%) من العينات المأخوذة من التهابات جيوب اللثة وبعد عزلها وتشخيصها بالاعتماد على نموها في الوسط الخاص بها FSM تحت الظروف اللاهوائية واجراء الاختبارات الكيمياء حيوية عليها وجدنا ان كل العزلات كانت متغايرة النتيجة تجاه اختبار اليوريز والكل حساسة للمضادات الحيوية (IPM_{10}) بنطاق تثبيط (42.7 ملم/قرص)، (CIP_{10}) (27.2 ملم/قرص) و (E_{15}) (25.0 ملم/قرص) على التوالي بينما أظهرت مقاومة لبقية المضادات الأخرى. وجد ان كل الأنواع التابعة لجنس *Fusobacterium* spp. تحت الدراسة حاوية على الجين 16S rDNA (حجم 360 زوج قاعدي) بينما وجد اثنان من العزلات تمتلك الجين *fadA* (بحجم 232 زوج قاعدي) المسؤول عن قدرتها على تكوين الاغشية الحيوية.

الكلمات المفتاحية: تكوين الاغشية الحيوية، الجين *FadA*، *Fusobacterium* spp.، 16S rDNA