ROLE OF MAJOR FIMBRIAE (FIM A) IN ADHESION AND BIOFILM FORMATION OF LOCAL ISOLATE *PORPHYROMONAS GINGIVALIS*[+]

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Abstract:

Background: Many previous studies indicate the role of the fimbriae of *P.gingivalis* in biofilm formation to biotic and abiotic surface *P.gingivalis* without fimbriae cannot gain entry inside epithelial cell. *P. gingivalis.* Fimbria-deficient mutants have a reduced ability to invade human gingival epithelial and human oral epithelial cell line.

Objetive:To study the role of fimbria (fim A) isolated from *P. gingivalis*, local isolates in adhesion to buccal epithelial cells.

Patients and methods:Thirty eight patients with chronic periodontitis enrolled for this study. Six (15%) isolates was identified as *Porphyromonasgingivalis*. Major fimbriae with MW (41KDa) were isolated from P5 isolate, partial purified was done byGoulbourne& Ellen. *P. gingivalis*(P5) was grown anaerobically,fractions were collected and proteins were measured at 280nm, Fraction with the same profile were used (Amicon,Grace& Co.,Danvers,Mass). Protein filtration was analyzed by procedure of Laemmli.

Result: Pretreatment buccal cells with partial purified fimbriae reduce the number of attached bacteria in a dose dependent manner, the highest effect was seen at the fambrial concentration 40µ (8±2)bac/cell while the lowest effect was at the concentration 10 µ(29±3)bac/cell. Anti- fimbriae reduced bacterial number attached to buccal cells and inhibit biofilm formation. Effect of fimbriae on lymphocyte and neutrophils viability was examined by trypan blue.

Conclusion: Specific antibodies against fimbriae could be used as a therapeutic agent against an aggressive type of these bacteria and reduce its pathogenicity. This approach therefore, serves as a new means to fight infectious diseases.

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دور الخمل الرئيسية (نوع اي) في عملية التصاق وتكوين الغشاء الحيوي لعزلة محلية لبكتريا *PORPHYROMONAS GINGIVALIS*

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المستخلص:

الاهداف: لمنع الاصابة بامراض اللثة المزمنة وتطورها المتسببة عن البكتريا Porphyro*monas* ***gingivalis*** اللاهوائية والسالبة لصبغة كرام.

خلفيات البحث: معظم الدراسات السابقة تشير الى دور الخمل الرئيسية لبكتريا ال***P.gingivalis*** في تكوين الغشاء الحيوي للسطح الحيوي واللاحيوي كما انها لاتتمكن من الدخول الى داخل الخلايا الطلائية المبطنة للفم بدون الخمل وكذلك وجد ان ال***P.gingivalis*** المحوره والخالية من الخمل تقل قابليتها لغزو الطبقة الطلائيه للثة الجنس البشري وخط الخلايا الطلائية لفم الانسان.

المرضى وطريقة العمل: ثمانية وثلاثون من المرضى الذين يعانون من التهاب اللثة المزمنة خضعوا لهذه الدراسة. شخصت ستة (15٪) عزلات على انها بكتريا ***Porphyromonas gingivalis***. تم عزل خمل رئيسية وبوزن (41KDa) MW من عزلة P5 وتمت التنقية الجزئية بواسطة كولبورني والين بعد اخذ عزلة من (p5) ***P.gingivalis*** وتنميتها لاهوائيا وعزل البكتريا من الوسط الزرعي .جمعت اجزاء البروتينات وقيست ب nm 280. جزء من نفس التشكيل ازيلت عنه الاملاح وركزت بواسطة دايفلو واي ام 30 Ultra-filtration membranes (Amicon, Grace& co., Danvers Mass)

البروتينات المترشحة حللت بواسطةاستخدام The SDS PAGE procedure of Laemmli[9].

النتائج: وجد ان المعالجه المبكرة لخلايا الشدق بالخمل الرئيسيه المنقات جزيئيا قبل اختبار الالتصاق يؤدي الى تقليل عدد الخلايا البكتيريه الملتصقه بخلايا الشدق وبطريقه تتناسب وتركيز الخمل الخلايا الشدق . فقد وجد اعلى تثبيط عند التركيز40 مايكرو كرام وكان عدد الخلايا الملتصقه يساوي(8±2) بكتريا لكل خلية بينما اقل تاثير كان عند التركيز 10 مايكرو كرام فقد كان عدد الخلايا(29±3) بكتريا لكل خلية. اضداد الخمل لها تاثير تثبيطي في عمليه التصاق البكتريا وتكوين الغشاء الحيوي بطريقة تتناسب وتركيز الاضداد. تم دراسة تاثير الخمل الرئيسيه على حيوية الخلايا اللمفاويه والعدله باستعمال صبغه تريبان الزرقاء.

الاستنتاجات: بالامكان استعمال الاضداد المتخصصة ضد الخمل كعوامل علاجية ضد هذه الانواع من البكتريا الضارة وتقليل امراضيتها. هذا يعد احدى الطرق الحديثة للقضاء على الاصابة بالامراض .

Introduction:

*Porphyromonas gingivalis*Gram negative anaerobes, is a major etiological agent in the initiation and progressive of severe form of periodontal disease [1].This opportunistic

pathogen colonized the subgingival region facilitated by the ability to adhere to an available oral surface such as epithelial cells, matrix protein, bacteria already established a biofilm on the tooth and the epithelial cell surface, binding to all these substrates may be mediated by bacterial fimbriae[2].Fimbriae play an important role in the initial interaction between the bacteria and the host. Available evidence suggests that fimbriae are important in adhesion to epithelial cells or human buccal epithelial cells[3].

Many previous studies indicate the role of the fimbriae of *P.gingivalis* in biofilm formation to biotic and abiotic surface while,*P.gingivalis* without fimbriae cannot gain entry inside epithelial cell [4].*P. gingivalis* fimbria-deficient mutants have a reduced ability to invade human gingival epithelial and human oral epithelial cell line [5].Afimbriated variants of*P.gingivalis* have also been shown tohave a diminished capacity for adherence to oral epithelialcells in an invitro assay [6]. Two distinct fimbriae are present on the surface of *P. gingivalis* cells, Major fimbriae are long, peritrichous,with MW about 40-44KDa (FimA, fimbrillin) and is encoded by the *fimA* gene and athin, short secondary fimbrial structure, termed minor fimbriae, is composed of a 67-kDa protein encoded by the *mfa1* gene [4].

Both major and minor fimbriae appear to contribute to the pathogenicity of *P. gingivalis* [1]. Our study clarifies the role of fimbriae of local*P. gingivalis*isolate in adhesion to buccal epithelial cell and the effect ofanti-fimbriae in prevention this attachmentand inhibition of biofilm formation.

Materials and methods:

Sample collection and bacterial identification:

Thirty eightadult patients with chronic periodontitis (from Dental care center /Baghdad) (January – September2015), were enrolled in this study. Periodontal pocket with 4-6mm, were included, isolation was done accordingto[7]. After isolation of dental caries bacteria from dental plaque with the cotton roll, supragingival was removed with curette and gingival fluid was collected by insertion of sterile paper points into pocket for 30 seconds.Paper points were then transferred in transfer media (trypticase soy broth /Difco) supplemented with 1µg/mL of yeast extract, 5µg/mL of hemin and 1µg/mL vitamin K. The samples then streak on trypticas soy agar supplemented with5%SRBs(Sulfate reducing bacteria's), 5µg/mL of hemin and 1µg/mL vitamin K, Incubation under anaerobic condition(with 80%N_2,10%CO_2and10%H_2(BD/BD GasPak) at 37° C for 5days [1]. All isolates form black colonies inoculated on brain heart infution agar (BHI) and on blood agar plate after one week incubation. The bacteria were diagnosed on the basis of color, size, shape, staining and biochemical tests.

Isolation and purification of fimbriae from *P.gingivalis*

Isolation of fimbriae from *P.gingivalis* was done as previous described byGoulbourne& Ellen [8]isolate*P.gingivalis*(**P5**) was grownanaerobically all isolates form black colonies inoculated on brain heart infusion agar (BHIA) and on blood agar plate after one week incubation.In two liter of trypticase yeast extract broth supplemented with 5µ/mL hemin and 1µ/mL vitamin K. Bacteria were harvested from broth culture and suspended in 20mM TrisHCl containing 0.15M NaCl and10mM $MgCl_2$, pH 7.4, byrepeated pipetting. The total volume of resuspending buffer was equal to 10% of culture medium volume. The suspension was stirred magnetically for 30min. then centrifuged at 7000 rpm for20min.at 4℃, supernatant containing fimbriae was retained for further study. Ammonium sulfate was added gradually to the supernatant which contain fimbriae to reach40% saturation with continuously stirring at room temperature for50 min. and allowed to stand overnight at4℃.

Precipitate protein were collected by centrifugation15000 rpm at 4℃ for30 min. and resuspeneded in a small volume of 20 mMTrisHCl, pH 8.The suspension was dialysate against Tris buffer for48 hour at 10℃, the dialysate was clarified by centrifugation at 10000 rpm for for15min.at 4℃ and the sample was applied on column of Sepharose CL-6B column (Pharmacia,Sweden) equilibrated with Tris buffer. The column was washedwithTrisbufferandelutedwith a linear gradient of 0 to 0.3M NaCl followed by a stepwise gradient of 0.3 to 1M NaCl. Fractions were collected and proteins were measured at280nm, further analysis was done by sodium dodecylsulfate-poly acrylamide gel electrophoresis. Fraction with the same profile were pooled,desalted and concentrated by Diaflo YM 30 ultra-filtration membrane (Amicon,Grace& Co.,Danvers,Mass)Protein filtration were analyzed by the SDS PAGE, procedure of Laemmli was used [9]. Sample was added to an equal volume of dissociating sample buffer and heated to 100℃ for 10min.Polyacrylamide (12. 5%) were loaded with 20mL contain0.25µg of protein, and a constant voltage of 175 v was applied for45 minute. Molecular weight was determined according to low molecular weight standard proteins, all the process have been done in the college of science Lab., ELISA kit (Biolegend,Sandiego, USA).

Anti-fimbriae antisera:

At the Immunity Lab. the college of science – Baghdad university. Previously described by [8],two males of New Zealand white rabbit were inoculated with 50 µg purified fimbriae mixed with Freund's adjuvant intramuscularly as a first inoculum. The two other booster injections with the same quantity .50 µg without adjuvant was administrated with period two weeks between each injection .bleeding for antisera was done after two weeks of the last booster injection. The existence of anti-fimbriae was examined by the Ouchterlony gel diffusion method.

Salivacollection:

Saliva was collected from two healthy adults,without any medication during two months before the study and had no oral disease. Stimulated saliva was collected by chewing paraffin gum for five minutes and expectorating into a sterile plastic cup. The saliva was immediately clarified by centrifugation at 10.000 rpm for 20 min. at 4℃ and filtrate using membrane filters (pore size 0.22µm).

Preparation of epithelial cells:

Buccal epithelial cells from healthy adults were collected by vigorous swabbing of the buccal mucosa with a sterile, cotton tipped swab. In 5 mL of PBS and washed three times with PBS by centrifugation (I0min.at 2000 rpm) to get rid of unattached bacteria. The cells were then

suspended in PBS at concentration of 1×10^5cells per mL, as determined by microscopic count [9].

Bacterial adhesion assay:

By using cultureplate as described by [10],*P.gingivalis* was added to each well contain buccal epithelial cells pretreated with saliva or with PBS as control at the ratio of(bacterial cells to epithelial cell) 100: 1, incubated anaerobically at 37℃ for1h. Finally, cells were washed stringently three times to remove loosely attached bacteria with PBS, fixed with methanol for20min, and stained with Giemsa solution. The number of bacteria adhering to epithelial cell was counted (on 50 cells) microscopically.The mean number of bacterial cells per epithelial cell was expressed.

Biofilm assays:

Biofilm formation was assayed using the protocol of O´Toole [11]. Briefly an overnight culture was diluted with fresh Brain Heart Infusion broth (BHI) to obtain 10^8 CFU/ml. Two ml of bacterial suspension was added to each tube pretreated with sterile saliva by adding 1mLof sterile saliva to each tube and incubated for 2hr at 37° C to get salivary pellicle, saliva was removed by Pasture pipette),incubatedanaerobicallyat 37℃ for36hr.the broth was removed.The resulting biofilms were washed, stained with 0.1% crystal violet, and then absorbance measurement at 550nm.

Specific fimbriaeantibodies effect on biofilm formation was done by using microtiter plates pretreated with saliva.

Effect of fimbriae on lymphocyte and neutrophil viability: lymphocyte was isolated according to method [12] and neutrophils according to[13]separately, prepared at concentration $1\times10^{5.}$ 0.5mLof lymphocyte or neutrophilsuspension was added to 0.5mlof fimbriaeantigen to obtain different concentration (10, 20, 40, and 80) incubated for 1hour then stained with trypan blue dye to detect cells viability

Inhibition bacterial adherence by specific antibodies: The procedure was done according to the method previously described by wingberg*et al.*[6]. Different dilutions from 1/10 to 1/100 of fimbriae antiserum was added to the bacterial suspension at the ratio 1/1 mixedforone hour at 37℃ suspension was washed twice with PBS, followed bacterial adhesion assay.

Statistical analysis: was done according to the computer program SPSS.Data are expressed as mean±SE, T test was used to determine the difference between the groups statistically significant.

Results and discussion:

Of thirty eight patients with chronic periodontitis enrolled in this study, *P.gingivalis* was isolated from 6(15.7%) of the total samples. In 28(73.6%) of the patients, depths of periodontal pocket were 4-6mM and majority of them 31(81.5%) had hemorrhage during sampling. Study by [14] showed that P.gingivalis represent 23% of total bacteria isolated from periodontitis. While [15] was found 28.7%in periodontal patients and36.7%in healthy subjects and[16]found25% of the healthy subjects and 79% in periodontitis group by using PCR and 16r DNA. From previous studies there was variation in detection rate of*P. gingivalis*and this may be due to the differences in individuals in the communities under (enrolled) study, detection methods, PCR more sensitive and specific.

Microscopic examination and biochemical tests: *P.gingivalis*is anaerobic Gram negative coccobacilli to small rods shaped. Non spore former,non-motile. Four from six clinical isolates showed smooth colonies and two isolates were identified rough. All isolates form black colonies on brain heart infusion agar (BHI) and on blood agar plate after one week ofincubation. Formation of black spots colonies was due to its participation in iron transport, the way it does by using a hemin as adeviceto help.[17]All isolates showed beta hemolysis onblood agar plates. All*P. gingivalis*isolate studied produced an active hemolytic activity during growth, with maximum activity occurring in late-exponential-early-stationary growth phase. All six clinical isolates of *P.gingivalis* could proteolysis milk.

Through this study, It was observed that this local isolate bacteria possess a wide range of virulence factors qualify it to be important pathogen in periodontitis.

Fimbriae of *P.gingivalis* were isolated from supernatant of *P.gingivalis* after 36 hr. of incubation when the growth reachesthe exponential phase. by using anion exchange chromatography (sepharose 6b),most of the fimbriae were eluted with 0.15M NaCl from fraction 120 to 300 these fractions pooled concentrated and applied onSDS PAGE Fimbriae an obvious 41KDa band on 12% polyacrylamide SDS gel showed in fig.1.this fimbriae with MW 41KDa, it was called major fimbriae was different from minor fimbriae which has MW 67KDa.

Chemical analysis of fimbria preparation showed highly purified with approximately 96% protein by using method[9] and 2.8%carbohydrate by method [18]. Nucleic acid was not detectable according to method [19]. Previous studies by [3]showed that major fimbriae of *P.gingivalis* was eluted at the fraction from 290 to 380 have molecular weight 43kDa. [20]Clarified that major fimbriae have 41KDa.Blood adhesion on buccal epithelial cells.There are many various methods used in previous studies to enumerate bacterial adherence to tissue cells. These include direct counting using a light microscope, plate counting, and radiometric assay. It was used direct methods because it distinguished between adherent bacteria (outside)and invades bacterial cells (inside) epithelial cells. Differential staining technique by Gmisastain was employed, examination of fixed, air dried slide microscopically.

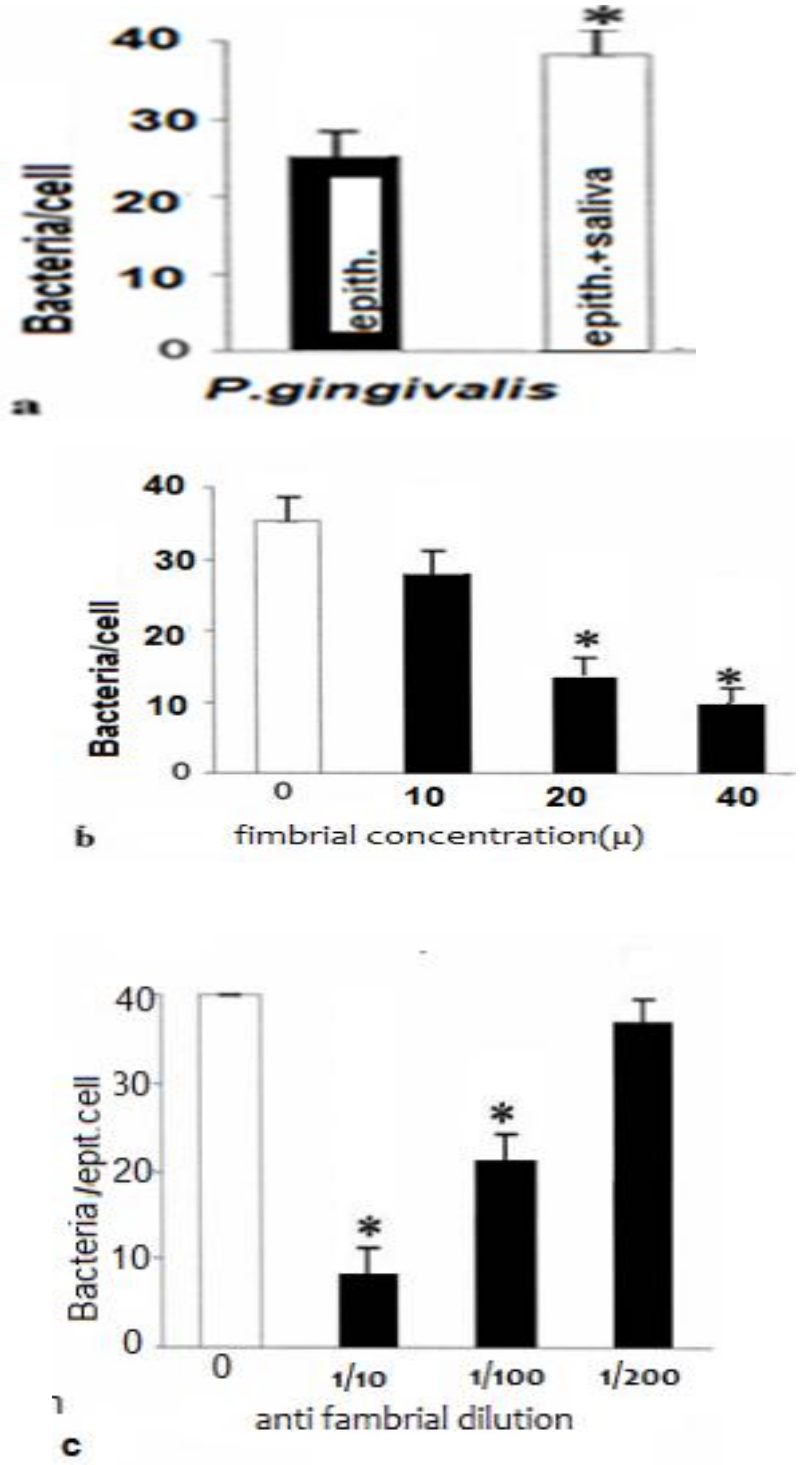

Fig 1: Effect of a-saliva, b-partial purified fimbriae and c- anti- fimbriae antibodies on bacterial attachment to buccal epithelial cell. P≥0.05considered as a significant difference. Bars represent standard error (n=4)

Fig. 1a.Showed the effect of saliva on bacterial attachment to buccal cells. Pretreatment of buccal cells with saliva for one hour at 37 °C, cause the formation of salivary pellicle which had a significant effect in developing of *P. gingivalis*adhesion to buccal epithelial cellsand

biofilm formation. This study is agree with that done by [4] which indicate that *P.gingivalis* not very competent to adhere to surface and colonize it without the help of either saliva or other bacterial species. These finding are in agreement with [2] which examined the adherence of*P.gingivalis* to abiotic hard surface. It was clear that salivary pellicle provides the necessary receptors allowing bacterial attachment and invasion thereafter.

Preincubation of partial purified *P.gingivalis* fimbriae with buccal cells before adhesion assay decrease bacterial number binding to these cells fig 1b. Isolated fimbriae blocked receptor on buccal cell for fimbriae of *P.gingivalis*. This inhibitory effect was dose dependent manner and highest effect was seen at fimbriae concentration 1/10µg, average7 adherent bacteria/ epithelial cell when compared with control there was statistically significant at the level $p \leq 0.05$while 1/200 had no effect. Our study in agreement with [5].

Anti-fimbriae antibodies serum titer was found 240.Pretreatment *P.gingivalis*with different concentration of antibodies separately for one hour before adhesion assay decrease bacterial number that attached buccal epithelial cell in a dose dependent manner,this due to the reaction of antibodies with epitopes on bacterial fimbriae.The most effective concentration was 1/10 while 1/100 was less effect and the number of attached bacteria was 7and 22 respectively. There is no significant effect with concentration 1/200 at level of $P \leq 0.05$ compared with control [18]. Anti-fimbriae antibodies block the site of attachment on bacterial fimbriae cause adhesion inhibition.Our finding in agreement with (9)which showed that specific antibodies against native FimA protein can protect against *P.gingivalis.*There was an opinion to use monoclonal antibodies against Fim A as passive immunization against the bacterial infection and prevent binding onto oral surface [19].Effect of partial purified fimbriae on neutrophils and lymphocyte viability was shown in figure 2and 3. Effect of Fim A isolated from *P.gingivals* on isolated PMN and lymphocyte in vitro was dose dependent and lymphocyte was more affected than PMN. The mean value of viable count at different concentration (0,20,40,80)µ for PMN and lymphocyte was (90.1,80±3.4, 58±2.1,40±2.4),(92,65,48,36)respectively. The results clarified significant differences between lymphocyte, PMN and the control; there was an inverse relationship between the fimbrial concentration and the cell viability, these results agree with (20) who referredthat the high concentration may causes osmotic shock which lead to kill the cells.

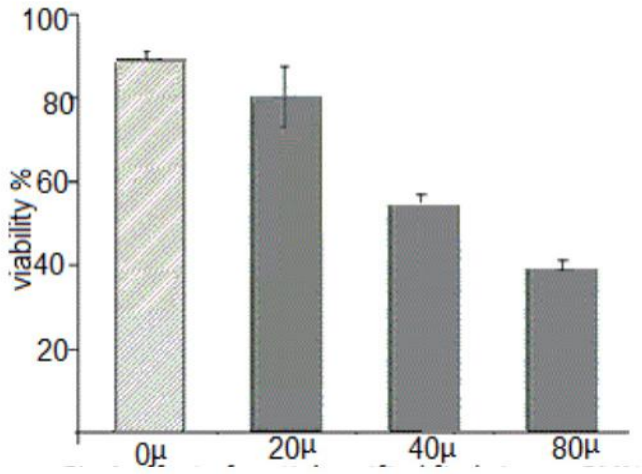

Fig.2. Effect of partial purified fimbriae protein concentration on PMN

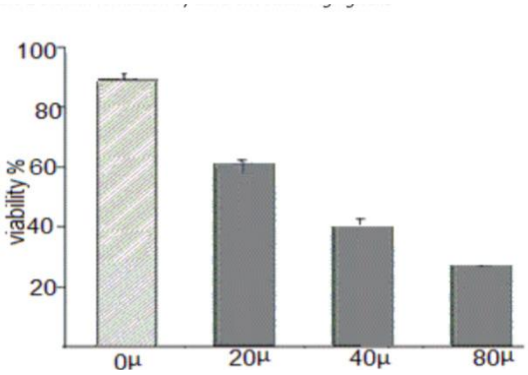

Fig.3. Effect of partial purified fimbriae protein on lymphocyte viability

Screening for biofilm formation: All six isolates of P.gingivalis were enrolled in this study. Different isolatesshowed a varied capacity in biofilm production.

Table 1Biofilm formation by different isolate of*P.gingivalis*

Bacterial isolates	absorbance at 550nm
P1	0.330±0.14
P2	0.267±0.02
P3	0.379±0.314
P4	0.280±0.461
P5	0.432±0.154
P6	0.185±0.22

As shown in table 1 the highest value of biofilm was 0.432±0.154 which beyond isolate P5 while the lowest value was 0.185±0.22which is beyond isolate P6, when experiment done by using crystal violet method and measuring absorbance at 550 nm[18]. This variation may due to difference in genotype,type II *fim* Ahave revealed their significantly greater adhesive and invasive capabilities as compared to other *fim*A type clone [21].

Studying the effect of anti-fimbrial antibody on biofilm formation was showna reduction in biofilm formation in a dose dependent manner; the highest effect was seen at the dilution 1/10(0.02±0.01) while the lowest effect was at dilution 1/80 (0.42±0.02). As shown in fig 4. Reduction in biofilm formation by specific antibodies due tothe blocking the site of attachment of the intact fimbriae on bacterial cell to the polystyrene microliter plate surface and preventing formation the biofilm.

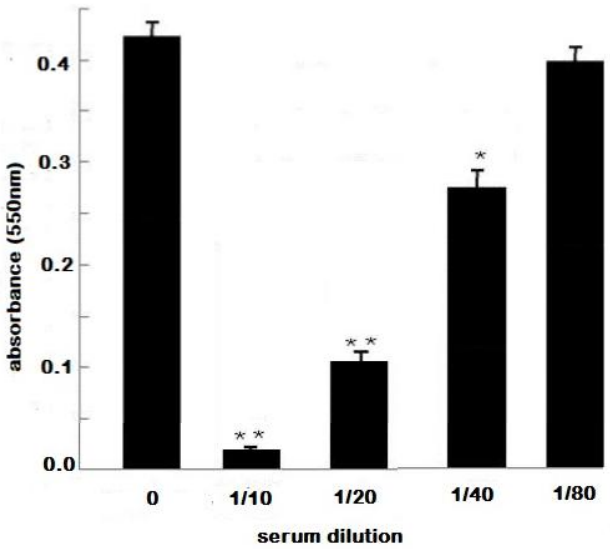

Fig.4Effect of anti fimbrial antibodies on biofilm formation

This study clarified the role of *P.gingivalis*fimbriae isolated from local isolate on attachment to thebuccal cells and biofilm formation on abiotic surface such as Polystyrene.Specific antibodies against fimbriae could use as a therapeutic agentagainst an aggressive type of this bacteria and reduce its pathogenicity.Thisapproach therefore, serves as a new means to fight infectious diseases.

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