Inhibitory effects of azithromycin on the adherence ability of *Porphyromonas gingivalis* ATCC 33277

Powen Kan,[1] Haruka Sasaki,[2] Keitaro Inaba,[2] Kiyoko Watanabe,[2] Nobushiro Hamada,[2]and Masato Minabe[1]

[1] Division of Periodontology, Department of Oral Interdisciplinary medicine, Kanagawa Dental University, Kanagawa, Japan

[2] Division of Microbiology, Department of Oral Science, Kanagawa Dental University, Kanagawa, Japan

Correspondence to: Masato Minabe

Division of Periodontology, Department of Oral Interdisciplinary medicine, Kanagawa Dental University, Kanagawa, Japan

82 Inaoka-chi, Yokosuka 238-8580, Japan

Phone & Fax: +81-468-22-8867

Email: minabe@kdu.ac.jp

Four figures and one table

Running Title: Inhibitory effects of azithromycin on the adherence ability of *Porphyromonas gingivalis* ATCC 33277

Summary of key findings:

Sub-inhibitory concentration of azithromycin effects on the adherence of *P. gingivalis*, through suppressing its fimbriae expression and hemagglutinin activity.

Background: *Porphyromonas gingivalis* is one of the major pathogens, and has a high detection rate in periodontal disease. Fimbriae and hemagglutinins are expressed by *P. gingivalis*, which play an important role in the adherence of the bacteria to periodontal tissue and biofilm formation. The aim of this study was to investigate the effects of sub-inhibitory concentrations (sub-MICs) of Azithromycin on the adherence of *P. gingivalis*, focus on the inhibition of fimbriae expression and hemagglutinin activity.

Methods: *Porphyromonas gingivalis* ATCC 33277 were incubated with the sub-MICs of azithromycin anaerobically at 37℃ by gentle shaking for 18 h. The bacterial cells were harvested, washed twice with PBS and separated by 12% SDS-PAGE and Western blotting. The Adherence assay and hemagglutinin Activity tests had done with the same culture.

Results: The results of SDS-PAGE indicated that the sub-MICs of azithromycin inhibited the 41-kDa protein expression and hemagglutinin activities. The clearly disappearance of 41-kDa protein expression and long fimbriae in 0.4 μg/ml, 0.2 μg/ml and 0.1μg/ml azithromycin were confirmed by western blotting and transmission electron microscopy. The adherence of *P. gingivalis* to HGEC was reduced by sub-MICs of azithromycin compared to the adherence levels without antibiotic.

Conclusion: These results suggested that sub-MICs of azithromycin can reduced the adherence of *P. gingivalis* to host cells, through inhibited the production of fimbriae and hemagglutinin activities. Furthermore, it can be used for oral biofilm infection treatment in periodontal disease.

KEY WORDS

Porphyromonas gingivalis; fimbriae; biofilm; antibiotic; periodontal disease

INTRODUCTION

Periodontal diseases, known as gingivitis and periodontitis, are characterized by gingival inflammation, periodontal pocketing, attachment loss, and then cause to tooth loss.[1] *Porphyronmonas gingivalis* (*P. gingivalis*) is a Gram-negative anaerobic bacteria that are recognized as a major pathogen in periodontal disease.[2] The virulence factors of *P. gingivalis* including lipopolysaccharide, fimbriae, hemagglutinins, capsule and gingiapain.[3] Especially, fimbriae and hemagglutinins are considered to play an important role in the adherence of *P. gingivalis* to periodontal tissue and biofilm formation.[4,5]

The *P. gingivalis* expresses two different forms of fimbriae on its cell surface, long fimbriae (FimA) encoded by the *fimA* gene and short fimbriae (Mfa1) encoded by the *mfa1* gene.[6, 7] The *fimA*-inactivated mutant (MPG1) strain of *P. gingivalis* was reported that loss of FimA resulted in reduced adherence to human gingival fibroblasts and epithelial cells, demonstrating that the FimA is the basic determinant of the adhesion ability of *P. gingivalis*.[4, 8] Another study also reported that △*fimA* mutant did not effect on *P. gingivalis* biofilm formation, and △*mfa1* mutant formed with thinner biofilm than *P. gingivalis* wild-type strain, suggesting that Mfa1 was found to be a positive regulator of *P. gingivalis* biofilm formation.[9, 10]

Hemagglutinins are bacterial surface protein that has been reported to regulate bacterial adhesion to the host cells, as well as agglutinates and hemolyzes erythrocytes.[5,] The Arg-gingipains (*rgp*) and Lys-gingipain (*kgp*) are particularly important for hemagglutinin activity of *P. gingivalis*.[11]Early studies reported the role of *fimA*/*mfa1* and gingipains (*rgp*, *kgp*) on biofilm formation. The *fimA* were shown to promote initial biofilm formation, *mfa1* and *kgp* were found to be negative regulators of micro-colony formation and bio-volume, and *rgp* likely controls micro-colony morphology and biovolume.[12] These interactions may play an important role between the bacteria and host tissue in the oral biofilm formation, which may induce the periodontal disease.

Azithromycin is belong to a unique class of macrolide antibiotic, differ chemically from other macrolide that it contains a nitrogen in the 15-membered macrolide ring.[13, 14] This makes azithromycin more resistant to acid breakdown giving it a longer half-life, better tissue penetration and greater activity against gram-negative organisms than other macrolide antibiotics.[13-15] Azithromycin has strong antibacterial activity against a wide range of bacteria by targeting the 50 ribosomal RNA of the organisms and inhibiting protein synthesis, including the common periodontopathic bacteria such as *P. gingivalis* and *Actinobacillus actinomycetemcomitans.*[15-18]

Azithromycin has a longer half-life (40-68 h) than Erythromycin (2-3 h) and

clarithromycin (4-7 h) after a single 500 mg oral dose.[14]Some studies tested the concentration of azithromycin in gingival crevicular fluid (GCF). Administered orally an initial dose of 500 mg azithromycin and another 250 mg doses on following two days. After 2d and 7d, the concentrations of azithromycin in blood serum were 0.22 ± 0.02 and 0.04 ± 0.01 mg/L, respectively. The concentration in GCF were 8.82 ± 1.25 and 7.38 ± 1.15 mg/L.[19] Another study reported that concentration of Azithromycin in GCF were 7.3 mg/L on day 2 and 2.5 mg/L on day 15, respectively.[20] The concentration of azithromycin in GCF is higher and more stable compared to serum levels and this made it a potential topical agent for the periodontal treatment.[13, 19-21]

A numbers of studies have exhibited immunomodulatory properties of macrolide antibiotics that are used in the infection disease, including diffuse panbronchiolitis and chronic obstructive pulmonary disease.[22, 23] Early studies have also supported the clinical and microbiological advantages of systemically administered azithromycin as an adjunct to periodontal treatment.[24-28] Hence, azithromycin with both antimicrobial action as well as anti-inflammatory or immune modulatory function, could be valuable used in the periodontal treatment.

The pathogenesis of periodontal disease involves the interplay of microbiota present between the biofilm and the host responses.[29]In recent year, there have been significant

advances in the understanding of periodontal disease, but little has change in terms of treatment for periodontal disease. The mainly treatment still using mechanical debridement to reduce the subgingival plaque.[30]

Azithromycin is effective against *P. gingivalis* biofilm at subinhibitory concentrations (sub-MICs).[31] However, the mechanism of sub-MICs of azithromycin against *P. gingivalis* biofilm has not been clarified.

Therefore, in this study, we assessed the effects of sub-MICs of azithromycin on the adherence of *P. gingivalis* to host cell in vitro, focus on the inhibition of fimbriae expression and hemaggluinating activity.

MATERIALS AND METHODS

Bacterial strain and antibiotic

The bacterial strain used was *P. gingivalis* ATCC 33277. *P. gingivalis* were grown at 37°C under anaerobic condition (15% CO_2, 15% H_2 and 70% N_2) in brucella broth supplemented with 5 mg/ml yeast extract, 5 μg/ml hemin and 0.2 μg/ml vitamin K_1. Azithromycin was purchased from Sigma-Aldrich Company and dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml.

Determination of the minimum inhibitory concentration (MIC)

P. gingivalis were incubated in Brucella broth for overnight and then adjusted with brucella broth to Optical density OD_{550} = 0.6. The concentrations of azithromycin were prepared by using 2 fold dilution method in 48-Well plate. Each volumes (500 µl) of the bacteria culture and antibiotic were prepared in 48-well plate and incubated anaerobically overnight at 37℃. MIC was recorded as lowest concentration that inhibited the visible growth of *P. gingivalis*.

SDS-Page and Western blotting

The *P. gingivalis* were incubated with the 1/2, 1/4, 1/8 MIC concentration of Azithromycin in Brucella broth by gentle shaking 18 h anaerobically at 37℃. After 18 h, the bacterial cells were harvested by 12,000 rpm for 15 min. After removed the supernatant and washed with the PBS twice. The samples were stained with coomassie brilliant blue R-250, heated at 100℃ for 10 min. The samples were applied to 12% polyacrylamide slab gels with 4% stacking gels and electrophoresed at a 20 mA constant current for 1 h.

After electrophoresis, the separated gel were transferred to a Polyvinylidene difluoride

membrane (BIO-RAD, USA) at 200 mA for 1h, and blocked membranes with TBS (20 mM Tris-HCl pH 7.4, 0.5 M Nacl) containing 3% bovine serum albumin (BSA) and 0.05% Tween at RT for 30 min (shaking). Then membrane were then incubate with antibody specific for *P. gingivalis* ATCC 33277 41-kDa fimbriae for overnight, washed in TBS-Tween, incubated for 2 h with anti-mouse IgG biotin conjugate, and then visualized by a substrate solution of 4-chloro-1-naphthol. The reaction was stopped by immersing the membranes in distilled water and dried.

Transmission Electron Microscope

The bacteria cells were harvested by 9000 rpm for 2min, washed by reduced transport fluid (RTF) twice and resuspended in 200 µl RTF. Nisshin support films (200 mesh) were made hydrophilic by Ion Sputtering. 10 µl of the cell suspension were applied on the support film and negatively stained with 2% uranyl acetate for 1 minute. The films were dry at room temperature for one day and examined with a JEM-1220 electron microscopy (Nippon Denshi Co., Tokyo, Japan) at 80 kV.

Hemagglutinating Activity assay

The hemagglutinating activity of the *P. gingivalis* was assayed using sheep

erythrocytes in 96-well round-bottomed microtiter plates. Sheep erythrocytes were washed and suspended to a final concentration of 2% in PBS. The bacteria cells were centrifuged, washed with PBS twice and adjusted to OD_{550} =1.0, and 50 μl aliquots were 2-fold step diluted in 96-well plates with PBS. Each well were mixed with 50 μl 2% sheep erythrocytes, incubated at RT for 1.5 h and the hemagglutinating activities of the test samples were determined (Hemagglutination was determined by visual inspection).

Adherence assay

The human gingival epithelial cells (HGECs) were obtained from dental patients. Adherence of HGECs by *P. gingivalis* was assessed as follows. The *P. gingivalis* cultures were centrifuged after incubated with sub-MICs of azithromycin, washed with KGM, and re-suspended in KGM at a final concentration of 10^9 cells/ml. Bacterial suspensions were added to confluent HGECs monolayers at a multiplicity of infection (MOI) of 100 and then incubated at 37℃ in 5% CO_2 for 90 min. after incubation, unattached bacteria were removed by sterile PBS twice. HGECs were lysed in 1 ml of sterile distilled water per wells and incubated for 30 min. Lysates were serially diluted, placed on Brucella blood agar plates and incubated anaerobically at 37℃ for 7 days.

The experimental procedures, were approved by the Ethical Committee of Kanagawa Dental University (approval no. 455).

RT-PCR

RNA was recovered from the cultures of *P. gingivalis* control strain and *P. gingivalis* exposed to sub-MICs of azithromycin. The bacteria cells were harvested, and mixed with ISOGEN following the manufacturer′s instruction. And The primers were the following: for 16S rRNA, 5′-CAATCGGAGTTCCTCGTGAT-3′ and 5′-TGGGTTTAAAGGGTGCGTAG-3′; for *fimA*, 5′-AATTCGAATCGTGCTTTTGG-3′ and 5′-GTCTTGCCAACCAGTTCCAT-3′; for *mfa1*, 5′-GAAAGTGCTGCTGGTAG-3′ and 5′-CAGATGGGTTGTTGCTCA-3′; for *rgpA,* 5′-GCCGAGA TTGTTCTTGAAGC-3′ and 5′-AGGAGCAGCAATTGCAAAGT-3′; for *rgpB*, 5′-CGCTGATGAAACGAACTTGA-3′ and 5′-CTTCGAATACCATGCGGTTT-3′; for *kgp*, 5′-CGCTGATGAAACGAACTTGA-3′ and 5′-CGCTGATGAAACGAACTTGA-3′; for *hagA,* 5′-ACAGCATCAGCCGATATTCC-3′ and 5′-CGAATTCATTGCCACCTTCT-3′. The

amplifications were performed with an initial denaturation at 95℃ for 2 min, followed by 30 cycles of 94℃ for 30 s, 65℃ for 30 s, 72℃ for 1 min and a final extension at 72℃ for 2 min. The samples were analyzed by electrophoresis on 1.5% agarose gel.

Statistical analysis

The statistical analysis was performed using BellCurve for Excel (Social Survey Research Information Co., Ltd) and data were analysed using one-factor analysis of variance followed by Tukey's test. Differences were considered significant at $P < 0.05$ (**$P < 0.01$, *$P < 0.05$, NS, $P > 0.05$).All values in this study were presented as mean ± SD.

RESULTS

Minimum inhibitory concentration (MIC) of azithromycin for P. gingivalis

In vitro antibacterial activity of Azithromycin against *P. gingivalis* was shown by detecting the minimum inhibitory concentration of azithromycin. The MIC of azithromycin for *P. gingivalis* ATCC 33277 was record as 0.4 μg/ml. The sub-MICs of

azithromycin were prepared to 0.2 μg/ml (1/2MIC), 0.1 μg/ml (1/4MIC) and 0.05 μg/ml (1/8MIC) in Brucella broth.

Effects of sub-MICs of azithromycin on P. gingivalis fimbriae expression

The effects of sub-MICs of azithromycin on *P. gingivalis* fimbriae expression were obtained by SDS-PAGE. The results showed that sub-MICs of azithromycin reduced 41-kDa fimbriae expression, but there were no significantly effects on 67-kDa fimbriae expression compared with the control (Figure 2. A). At 4 μg/ml, 0.2 μg/ml and 0.1 μg/ml of azithromycin, the clearly disappearance of 41-kDa fimbriae protein expression were obtained by Western immunoblotting (Figure 1. B).

Effects of azithromycin on fimbriae structures of the cell surface

In order to confirm the results of SDS-PAGE and Western Blotting, fimbriae structures were observed on the cell surface of *P. gingivalis* by transmission electron microscope. The control strain produced long fimbriae structures on the surface (Figure 2. a), and those structures were completely disappear in 0.4 μg/ml, 0.2 μg/ml and 0.1 μg/ml of azithromycin. (Figure 2. b, c, d)

Adherence and Hemagglutinating Activity

The results of sub-MICs of azithromycin on the adherence of *P. gingivalis* to human gingival epithelial cells were showed that Control strain (3.48 ± 0.32%), 0.4 (0.95 ± 0.11%), 0.2µg/ml (1.57 ± 0.05%), 0.1µg/ml (1.85 ± 0.07%) and 0.05 µg/ml (2.84 ± 0.09%) of azithromycin to HGEC (Table 1). The Hemagglutinating abilities of *P. gingivalis* culture supernatants from sub-MICs of azithromycin compared to control group are presented (Figure 3). The hemagglutination titer of *P. gingivalis* control strain was observed at 1:8, and a twofold reduction were resulted in 0.1 µg/ml (1:2) and 0.05 µg/ml (1:2) of azithromycin. However, there were no hemaggluinating activities observed in 0.4 µg/ml and 0.2 µg/ml of azithromycin (Figure 3).

RT-PCR

RT-PCR was used to confirm the effects of sub-MICs of azithromycin on virulence genes expression of *P. gingivalis*. The 16S rRNA was used to ensure the same amount of total RNA from the samples. The table shows that there was no differences between them in terms of 16S rRNA, *mfa1* and *rgpB* genes expression. Comparing to the *P. gingivalis* control stain, (*fimA*, *rgp*, *kgpA, hagA*) genes expression were significantly

decreased by sub-MICs of azithromycin (Figure. 4).

DISSCUSSION

Periodontal disease is one of the most common chronic infection disease caused by a multispecies biofilm that colonize on the tooth surface or below the gingival margin.[1, 32] The first step of biofilm formation on oral surface involves the adherence of *P. gingivalis* to the periodontal tissue or other bacteria cells.[32] *P. gingivalis* has been described as a keystone pathogen, reflected in tissue colonization and destruction, interference with host defense systems, and also promote disease pathogenesis.[1, 33] Thus, Inhibit the adherence ability of *P. gingivalis* can be a therapeutic approach to control colonization (or re-colonization) to oral tissues.

Azithromycin showed good anti-biofilm activity against *P. gingivalis* biofilm at sub-MICs, but the mechanism of sub-MICs of azithromycin against *P. gingivalis* biofilm has not been clarified.[31]In present study, sub-MICs of azithromycin blocked the 41-kDa fimbriae protein expression and inhibited the FimA formation of *P. gingivalis.* Another important adhesion factor of *P. gingivalis,* hemagglutinin activity was also inhibited by sub-MICs of azithromycin. Inhibition of fimbriae expression and hemagglutinin activity of *P. gingivalis* resulted in reducing its adherence to HGECs. Hence, sub-MICs of azithromycin may have anti-biofilm effects on *P. gingivalis* biofilm

in vitro, through inhibited the fimbriae expression and hemagglutinin activity.

Previous studies have showed that *rgp*A *rgpB kgp* –inactivated mutan and *rgp*A *kgp hag*A –inactivated mutan of *P. gingivalis* have no hemagglutinin activity using sheep erythrocytes, indicating that hemagglutinin activity of *P. gingivalis* is caused by *rgp*A*, kpg* and *hag*A gene.[11]The same outcomes were shown in this study that, the inhibition of *rpgA*, *kgp* and *hagA* by azithromycin were resulted in reducing the hemagglutinin activity of *P. gingivalis*. However, Hamada N et al., have demonstrated that the *fimA* did not have directly connection with the hemagglutinin activity of *P. gingivalis*.[12]These findings indicated that inhibition of fimbriae expression or hemagglutinins activity to reduce the adherence of *P. gingivalis* to HGECs were independent, suggesting that fimbriae expression and hemagglutinin were inhibited by azithromycin at the same time.

Azithromycin exerts its antimicrobial activity by blocking the protein production of organism.

The 41-kDa fimbriae protein and *fimA* gene of *P. gingivalis* were inhibited by sub-MICs of azithromycin, but there were no effect on 67-kDa fimbriae protein and *mfa1* gene. The mechanism of azithromycin for inhibiting special target protein will be our future study.

From a microbiological perspective, the clinical efficacy of antibiotic should retain its concentration above the MIC level in order to obtain an antimicrobial effect. Azithromycin effects may be antibacterial or anti-inflammatory depend on the drug concentration. It is important for clinical periodontal treatment, since it can support a long-term therapy, longer dosing intervals and costs without a loss in effectiveness. Although the convention scaling and root planning usually stop the disease process, the loss of bone and periodontal infection continues in some patients. The bacterial pathogens can invade the periodontal tissue and re-colonize after debridement. Therefore, administration of azithromycin before and after the dental debridement can reduce the biofilm formation and re-colonization.

In conclusion, the current study have investigated the inhibitory effects of sub-MICs of azithromycin on *P. gingivalis* ATCC33277. Sub-MICs of azithromycin show a good inhibitory effects on the adherence of *P. gingivalis* to HGECs through interfering the 41-kDa fimbriae formation and reducing the hemagglutinin activity. Therefore, it can be used as a biofilm treatment for periodontal disease which is cause by *P. gingivalis*.

FOOTNOTES

REFERENCES

1. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev 1998;62:1244-1263.
2. Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Mol Oral Microbiol 2012;27:409-419.
3. Nakayama K. Molecular genetics of *Porphyromonas gingivalis*: gingipains and other virulence factors. Curr Protein Pept Sci 2003;4:389-395.
4. Hamada S, Amano A, Kimura S, Nakagawa I, Kawabata S, Morisaki I. The importance of fimbriae in the virulence and ecology of some oral bacteria. Oral Microbiol Immunol 1998 Jun;13:129-138.
5. Kozarov E1, Miyashita N, Progulske-Fox A., et al. Expression and immunogenicity of hemagglutinin A from *Porphyromonas gingivalis* in an avirulent Salmonella enterica serovar typhimurium vaccine strain. Infect Immun 2000;68:732-739.
6. Yoshimura F, Takahashi K, Nodasaka Y, Suzuki T. Purification and characterization of a novel type of fimbriae from the oral anaerobe *Bacteroides gingivalis*. J Bacteriol 1984; 160:949-57.

7. Yoshimura F, Murakami Y, Nishikawa K, Hasegawa Y, Kawaminami S. Surface components of *Porphyromonas gingivalis*. J Periodontal Res 2009;44: 1-12.
8. Hamada N, Watanabe K, Sasakawa C, Yoshikawa M, Yoshimura F, Umemoto T. Construction and Characterization of a *fimA* Mutant of *Porphyromonas gingivalis*. Infect Immun 1994;62:1696-1704.
9. Lin X, Wu J, Xie H. *Porphyromonas gingivalis* minor fimbriae are required for cell-cell interactions. Infect Immun 2006;74:6011-6015.
10. Kuboniwa M, Amano A, Inaba H, Hashino E, Shizukuishi S. Homotypic biofilm structure of *Porphyromonas gingivalis* is affected by FimA type variations. Oral Microbiol Immunol 2009;24:260-263.
11. Shi Y, Ratnayake DB, Okamoto K, Abe N, Yamamoto K, Nakayama K. Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. J Biol Chem 1999;274:17955-17960.
12. Duncan MJ, Emory SA, Almira EC. *Porphyromonas gingivalis* genes isolated by screening for epithelial cell attachment. Infect Immun 1996;64:3624-3631.
13. Amsden GW. Erythromycin, clarithromycin, and azithromycin: are the differences real? Clin Ther 1996;18:56-72.

14. Zuckerman JM, Qamar F, Bono BR. Macrolides, ketolides, and glycylcyclines: azithromycin, clarithromycin, telithromycin, tigecycline. Infect Dis Clin North Am 2009;23:997-1026.

15. Hirsch R, Deng H, Laohachai MN. Azithromycin in periodontal treatment: more than an antibiotic. J Periodontal Res 2012;47:137-148.

16. Pajukanta R. In vitro antimicrobial susceptibility of *Porphyromonas gingivalis* to azithromycin, a novel macrolide. Oral Microbiol Immunol 1993;8:325-326.

17. Hoepelman IM1, Schneider MM. Azithromycin: the first of the tissue-selective azalides. Int J Antimicrob Agents 1995;5:145-167.

18. Pajukanta R, Asikainen S, Saarela M, Alaluusua S, Jousimies-Somer H. In vitro activity of azithromycin compared with that of erythromycin against *Actinobacillus actinomycetemcomitans*. Antimicrob Agents Chemother 1992;36:1241-1243.

19. Lai PC, Ho W, Jain N, Walters JD. Azithromycin concentrations in blood and gingival crevicular fluid after systemic administration. J Periodontol 2011;82:1582-1586.

20. Gomi K, Yashima A, Arai T, et al. Drug concentration in inflamed periodontal tissues after systemically administered azithromycin. J Periodontol 2007;78:918-923.

21. Blandizzi C1, Malizia T, Senesi S, et al. Periodontal tissue disposition of azithromycin in patients affected by chronic inflammatory periodontal diseases. J Periodontol 1999;70:960-966.

22. Li H, Zhou Y, Jiang D, et al. Effect of azithromycin on patients with diffuse panbronchiolitis: retrospective study of 51 cases. Intern Med 2011;50:1663-1669.

23. Yamaya M, Azuma A, Takizawa H, Kadota J, Tamaoki J, Kudoh S. Macrolide effects on the prevention of COPD exacerbations. Eur Respir J 2012;40:485-494.

24. Haas AN, de Castro GD, Rösing CK. Azithromycin as an adjunctive treatment of aggressive periodontitis: 12-months randomized clinical trial. J Clin Periodontol 2008;35:696-704.

25. Haffajee AD1, Torresyap G, Socransky SS. Clinical changes following four different periodontal therapies for the treatment of chronic periodontitis: 1-year results. J Clin Periodontol 2007;34:243-253.

26. Fujise O, Miura M, Hamachi T, Aida Y, Nishimura F. Regenerative effect of azithromycin on periodontitis with different levels of gingival inflammation: three case reports. Aust Dent J 2014;59:245-251.

27. Smith SR, Foyle DM, Williams J, et al. A double-blind placebo-controlled trial of azithromycin as an adjunct to non-surgical treatment of periodontitis in adults:

clinical results. J Clin Periodontol 2002;29:54-61.

28. Nakajima T, Okui T, Yamazaki K6, et al. Microbiological and Clinical Effects of Sitafloxacin and Azithromycin in Periodontitis Patients Receiving Supportive Periodontal Therapy. Antimicrob Agents Chemother 2016;60:1779-1787.
29. Kornman KS. Mapping the pathogenesis of periodontitis: a new look. J Periodontol 2008;79:1560-1568.
30. Greenstein G. Periodontal response to mechanical non-surgical therapy: a review. J Periodontol 1992;63:118-130.
31. Maezono H, Noiri Y, Ebisu S., et al. Antibiofilm effects of azithromycin and erythromycin on *Porphyromonas gingivalis*. Antimicrob Agents Chemother 2011;55:5887-5892.
32. Ruijie H, Mingyun L, Richard LG. Bacterial interactions in dental biofilm. Virulence 2011;2: 435-444.
33. George H, Richard PD, Michael A. The Keystone Pathogen Hypothesis. Nat Rev Microbiol 2012;10: 717-725.

FIGURE LEGEND

Figure 1. Effects of sub-MICs of azithromycin (AZM) on *P. gingivalis* fimbriae expression.

(A) SDS-PAGE; (B) Western Blotting. Arrows indicate the fimbriae bands of FimA (41-kDa) and Mfa1 (67-kDa) of *P. gingivalis*. Lane 1.without AZM; lane 2. AZM 0.4 μg/ml; lane 3. AZM 0.2 μg/ml; lane 4. AZM 0.1 μg/ml; lane 5. AZM 0.05 μg/ml.

Figure 2. Examination of fimbriae structure on *P. gingivalis* cell surface by electron microscopy. The bacteria cells were harvested after exposed sub-MICs of azithromycin. (a) Without AZM; (b) AZM 0.4 μg/ml; (c) AZM 0.2 μg/ml; (d) AZM 0.1 μg/ml; (e) AZM 0.05 μg/ml, respectively. Bar, 0.2 μm.

Figure 3. Hemagglutinating activity of *P. gingivalis* after exposed to sub-MICs of azithromycin. The hemagglutinating activity of the *P. gingivalis* was assayed using sheep erythrocytes in 96-well round-bottomed microtiter plates.

Figure 4. The effects of sub-MICs of azithromycin on virulence genes expression of *P.*

gingivalis ATCC 33277.

Table 1. Adherence of *P. gingivalis* to human gingival epithelial cells. The percentages were means of triplicate experiments ± standard deviations. Tukey‘s comparison test, N=3, * : $p<0.05$, ** : $p<0.01$

Figure1

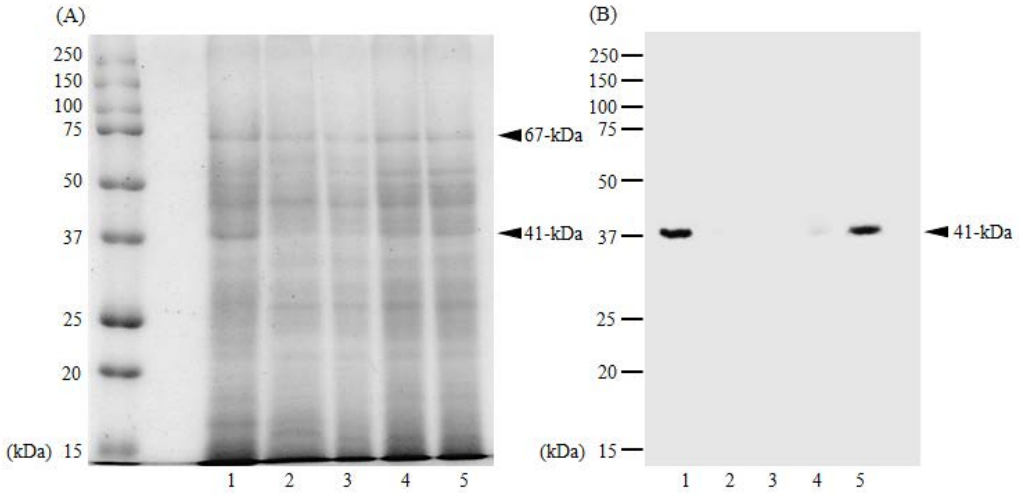
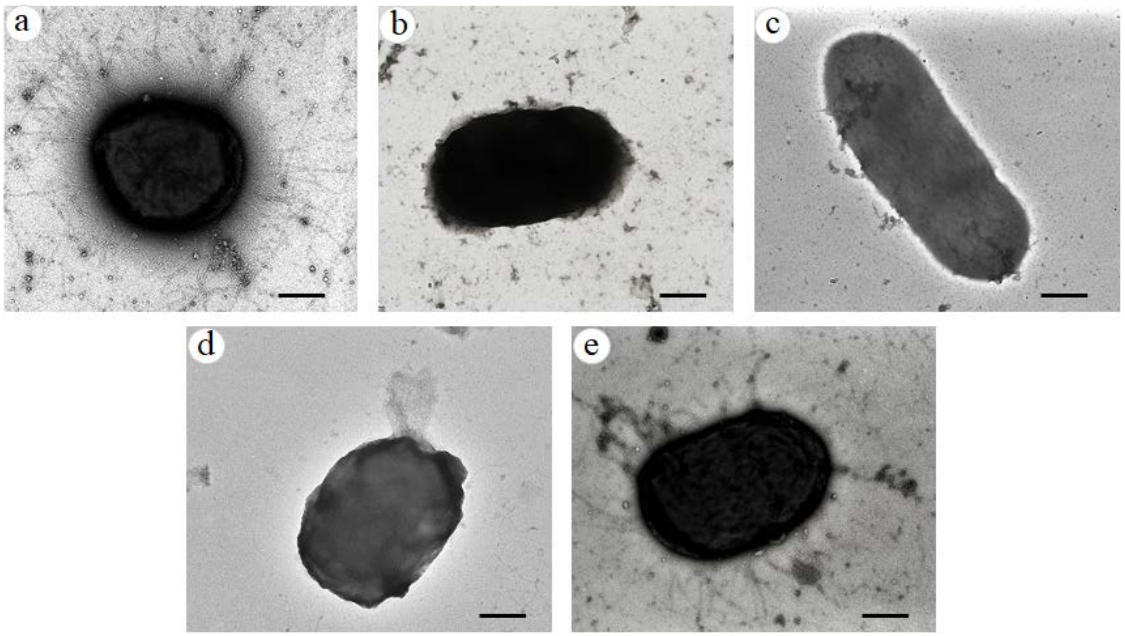

(A)
250
150
100
75
50
37
25
20
(kDa) 15
67-kDa
41-kDa
1 2 3 4 5
(B)
250
150
100
75
50
37
25
20
(kDa) 15
41-kDa
1 2 3 4 5

Figure 2.



a
b
c
d
e

Figure 3

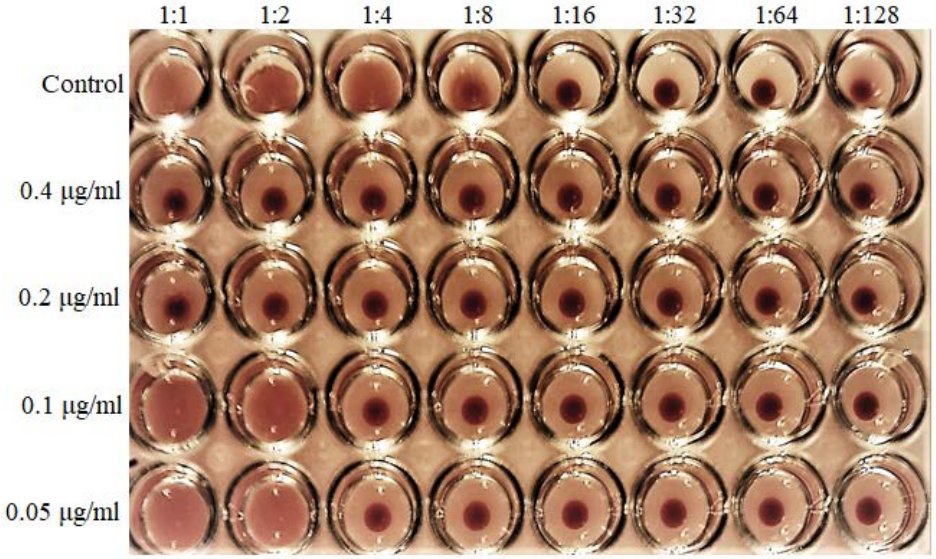

1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
Control
0.4 µg/ml
0.2 µg/ml
0.1 µg/ml
0.05 µg/ml

Figure 4

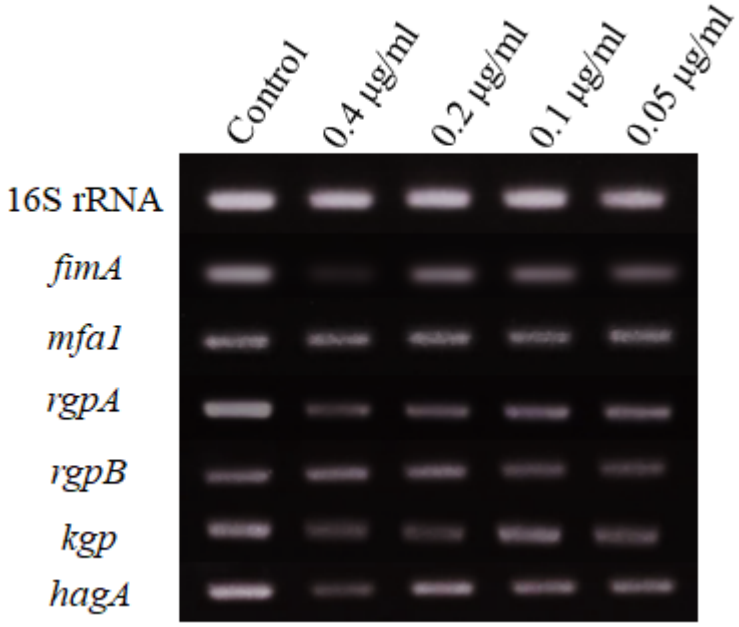
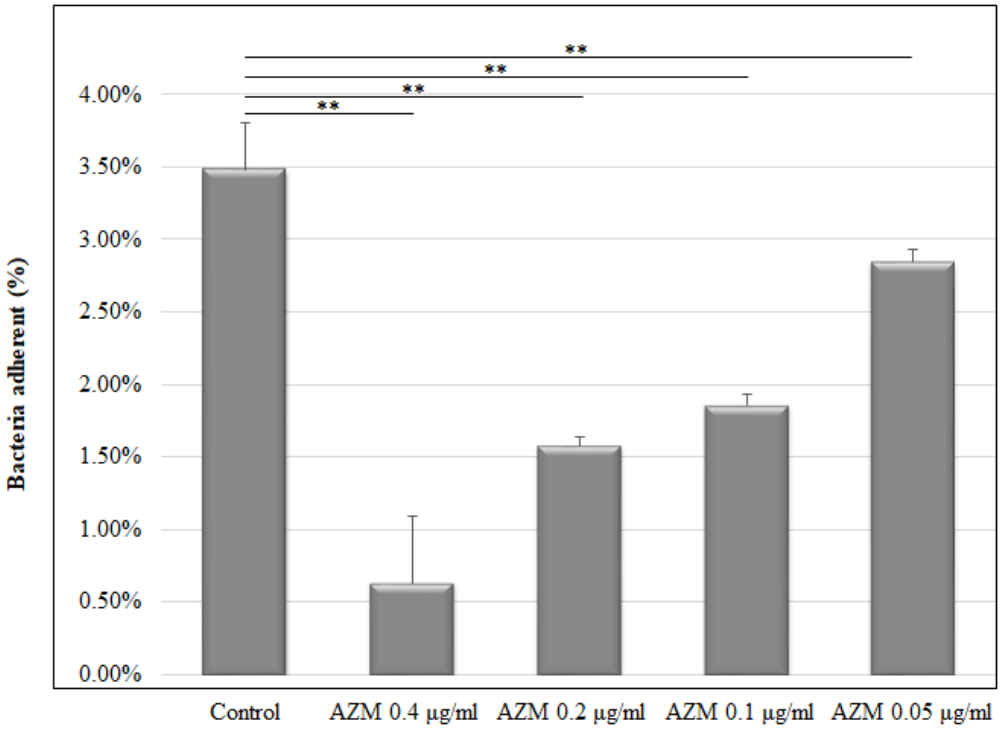

Control
0.4 μg/ml
0.2 μg/ml
0.1 μg/ml
0.05 μg/ml
16S rRNA
fimA
mfa1
rgpA
rgpB
kgp
hagA

Table 1


Bacteria adherent (%)
4.00%
3.50%
3.00%
2.50%
2.00%
1.50%
1.00%
0.50%
0.00%
**
**
**
**
Control
AZM 0.4 µg/ml
AZM 0.2 µg/ml
AZM 0.1 µg/ml
AZM 0.05 µg/ml