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Antibiofilm Effects of Azithromycin and Erythromycin on *Porphyromonas gingivalis*▽

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Antibiotic resistance of biofilm-grown bacteria contributes to chronic infections, such as marginal and periapical periodontitis, which are strongly associated with *Porphyromonas gingivalis*. Concurrent azithromycin (AZM) administration and mechanical debridement improve the clinical parameters of periodontal tissue *in situ*. We examined the *in vitro* efficacy of AZM against *P. gingivalis* biofilms. The susceptibilities of adherent *P. gingivalis* strains 381, HW24D1, 6/26, and W83 to AZM, erythromycin (ERY), ampicillin (AMP), ofloxacin (OFX), and gentamicin (GEN) were investigated using a static model. The optical densities of adherent *P. gingivalis* cells were significantly decreased by using AZM and ERY at sub-MIC levels compared with those of the controls in all the strains tested, except for the effect of ERY on strain W83. AMP and OFX inhibited *P. gingivalis* adherent cells at levels over their MICs, and GEN showed no inhibition in the static model. The effects of AZM and ERY against biofilm cells were investigated using a flow cell model. The ATP levels of *P. gingivalis* biofilms were significantly decreased by AZM at concentrations below the sub-MICs; however, ERY was not effective for inhibition of *P. gingivalis* biofilm cells at their sub-MICs. Furthermore, decreased density of *P. gingivalis* biofilms was observed three-dimensionally with sub-MIC AZM, using confocal laser scanning microscopy. These findings suggest that AZM is effective against *P. gingivalis* biofilms at sub-MIC levels and could have future clinical application for oral biofilm infections, such as chronic marginal and periapical periodontitis.

Biofilms are matrix-enclosed bacterial populations and are formed on inactive or bioactive surfaces (7). In medicine and many other fields, biofilms cause many problems, such as metallic corrosion and food contamination in processing. The characteristics of biofilms, such as the structure and composition of the extracellular matrix, have been reported (7). In medicine, bacterial biofilms cause chronic or refractory infections, so-called "biofilm infections," by forming films on medical devices, such as catheters or artificial joints. Biofilms formed on teeth or oral soft tissues are the main causes of oral biofilm infections, including caries, periodontitis, and mucosal disease.

Porphyromonas gingivalis is a Gram-negative obligate anaerobic bacterium, and it is detected throughout periodontal pockets in patients with severe chronic marginal periodontitis (34). *P. gingivalis* is also frequently detected from extraradicular biofilms, which are refractory infectious lesions that are located outside the root apex (31). The virulence factors of *P. gingivalis*, such as proteolytic activity, fimbriae, capsules, and hemagglutinating activity, have been described (29). *P. gingivalis* biofilms are resistant to chemotherapeutic agents, such as minocycline and metronidazole, although these agents are effective against planktonic *P. gingivalis* cells (33).

Generally, biofilms are resistant to antibiotics, and this plays an important role in the failure of chemotherapy for biofilm infections. It has been reported that azithromycin (AZM) has antibiofilm effects (14, 37). *Pseudomonas aeruginosa* biofilm is inhibited by sub-MIC AZM (14). Also, in *Haemophilus influenzae*, AZM suppresses monospecies biofilm formation and detachment of formed biofilms (37). More than 500 different bacterial species are known to form human oral biofilms (19). Most oral biofilm-forming species are opportunistic pathogens, and they seldom cause monospecies infections. It is unknown how AZM exerts its effect against oral biofilms.

AZM is a 15-membered macrolide antibiotic that is obtained from erythromycin (ERY) by inserting a methyl-substituted nitrogen into a 14-membered ring. AZM has two amine bases, which have been reported to give AZM excellent tissue transition and an exceedingly long half-life compared with those of other macrolide antibiotics (9). Oral administration of 500 mg AZM can maintain a high concentration in the target tissue after the blood level has decreased to an undetectable level (9, 11). AZM administration is once daily, which is different from other antibiotics, because of its unique characteristics, such as long half-life and tissue accumulation (9). AZM is effective mainly against Gram-positive bacteria; however, it has a wide spectrum and also shows efficacy against Gram-negative bacteria (18).

In periodontal treatment, a combination of AZM and mechanical debridement significantly inhibits regrowth of peri-

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odontopathic bacteria, such as *P. gingivalis* and *Tannerella forsythia*, and improves clinical features, such as periodontal pockets and clinical attachment levels (10). In respiratory medicine, low-dose, long-term ERY is an established treatment for diffuse panbronchiolitis (DPB) (21). The characteristics of this treatment show that ERY also has other effects apart from antimicrobial activity.

Sub-MICs of antibiotics are effective for the treatment of DPB or cystic fibrosis (8, 21, 42). Research on the efficacy of sub-MICs of antibiotics against bacteria or biofilms of various strains *in vitro* is progressing in many fields, such as pathogenicity, antibiotic resistance, and quorum sensing (39). It has been reported that sub-MICs of AZM inhibit expression of fimbriae of *P. gingivalis* (23). Also, sub-MICs of AZM and ERY inhibit *P. gingivalis* biofilm formation in static models (38). However, macrolides with 16-membered rings, such as josamycin or midecamycin, cannot inhibit *P. gingivalis* biofilm formation or mucoid alginate biosynthetic enzyme activity of *P. aeruginosa* (27, 38).

Therefore, in this study, we focused on the effects of macrolides with 15- and 14-membered rings, including AZM and ERY, on biofilms of four strains of *P. gingivalis*, at several concentrations, including sub-MICs, using two biofilm models.

MATERIALS AND METHODS

Bacterial strains and culture conditions. We used four strains of *P. gingivalis* with different *fimA* genes, which encode fimbrial proteins (2): strain 381 with type I fimbriae, strain HW24D1 with type II, strain 6/26 with type III, and strain W83 with type IV. They were incubated at 37°C under anaerobic conditions (90% N_2, 5% CO_2, 5% H_2) using Gifu anaerobic medium (GAM) bouillon (Nissui, Tokyo, Japan), supplemented with 5 μg/ml hemin (Sigma-Aldrich, St. Louis, MO) and 1 μg/ml menadione (Wako Pure Chemical Industries, Osaka, Japan), as previously described (28, 32, 34).

Antibiotics. We used the 15-membered macrolide AZM (Wako), 14-membered macrolide ERY (Wako), ampicillin (AMP; Sigma-Aldrich), new quinolone ofloxacin (OFX; Wako), and aminoglycoside gentamicin (GEN; Nacalai Tesque, Kyoto, Japan).

Antimicrobial susceptibility test. MICs of AZM, ERY, AMP, OFX, and GEN for planktonic *P. gingivalis* were determined in the range 0 to 640 μg/ml by using 96-well U-bottom microplates (Becton Dickinson, Sparks, MD) according to the standard method of the Japan Society of Chemotherapy (20). All assays were performed in triplicate.

***P. gingivalis* adherent cells by static model.** Adherent cells using a static model were formed on 96-well, flat-bottom plates as previously described by Azakami et al. (4). Each of four strains of cultured *P. gingivalis* was dispensed in 200 μl per well and incubated for 3 days at 37°C under anaerobic conditions. Antibiotics were added at the final concentrations of 3.9×10^{-3} to 640 μg/ml using a serial dilution method and incubated for a further 3 days. In the controls, hemin-and-menadione-supplemented GAM broth was added. Quantitative analysis of adherent cells was also determined by a previously described assay (4). After 3 days of cultivation, supernatants were removed and stained with 1% crystal violet (Sigma-Aldrich). Plates were washed three times with distilled water and decolorized with ethanol. Optical density (OD) was measured at 595 nm using a spectrophotometer (model 680; Bio-Rad, Hercules, CA), and the minimum biofilm inhibitory concentration (MBIC) was determined.

Biofilm formation and biological assay of the *P. gingivalis* flow cell biofilm model. Details of the *P. gingivalis* biofilm formation model using a modified Robbins device (MRD) and hydroxyapatite (HA) disks have been described previously (33). Four separate MRDs and 40 HA disks (10 disks/MRD), which were processed with saliva for 8 h, were prepared for each strain. *P. gingivalis* biofilms were formed by anaerobic perfusion of culture medium containing bacterial cells for 14 days. Culture medium that contained antibiotics at various concentrations (10, 1, and 0.125 μg/ml) was perfused through three separate MRDs for a 7-day period. In the control group, only culture medium was circulated. HA disks were removed aseptically from each MRD after exposure to antibiotics and examined using an ATP bioluminescence assay. The latter was performed using an ATP kit (AF-3X2; DKK-TOA, Tokyo, Japan) as described previously (3, 13, 33). After being aseptically sampled from each MRD, the specimens were washed and ultrasonicated in 1.5 ml distilled water at 4°C for 30 min to remove the *P. gingivalis* biofilms from the HA disks. After sample processing according to the kit manufacturer's instructions, bioluminescence was measured using an ATP analyzer (AF-100 or DF-10; DKK-TOA). Antibiotics were selected that inhibited *P. gingivalis* adherent cells at their sub-MICs in the static model.

Three-dimensional observations of *P. gingivalis* flow cell biofilms. To examine the effect of AZM on *P. gingivalis* biofilms three-dimensionally, flow cell biofilm samples on celluloid disks (Celltight; Sumitomo Bakelite Co., Tokyo, Japan) were prepared using an MRD as described above. Biofilm samples were stained with the Live/Dead BacLight bacterial viability kit L7007 (Invitrogen, Carlsbad, CA) for 15 min at room temperature. The samples were observed by confocal laser scanning microscopy (CLSM; LSM 510; Carl Zeiss, Oberkochen, Germany). Images were processed by image analysis software (Imaris; Bitplane AG, Zurich, Switzerland).

Changes in drug susceptibility by exposure to sub-MIC AZM on *P. gingivalis* biofilm-detached cells from the flow cell model. Culture fluid of *P. gingivalis* 381, HW24D1, 6/26, and W83 was perfused for 14 days using an MRD as described above and formed biofilms. Medium that contained 0.125 μg/ml AZM, which is sub-MIC, was perfused for 3 days, and biofilm-detached cells were collected from perfused cultures. Control samples were obtained from antibiotic-unexposed *P. gingivalis* biofilm-detached cells. *P. gingivalis* biofilm-detached cells exposed/unexposed to sub-MIC AZM were adjusted to 10^6 CFU/ml using GAM broth. After that, MICs for *P. gingivalis* biofilm-detached cells exposed/unexposed to sub-MIC AZM were determined using 96-well microtiter plates, according to the standard method of the Japan Society of Chemotherapy (20).

Statistical analysis. The significances of intergroup differences in the static model and flow cell biofilm model were analyzed using Student's *t* tests. *P* values of <0.01 and <0.005 were considered to indicate statistical significance for static and flow cell models, respectively. The minimum concentration that inhibited *P. gingivalis* biofilms significantly was designated MBIC in the static model.

TABLE 1. MICs of AZM, ERY, AMP, OFX, and GEN for planktonic *P. gingivalis* strains 381, HW24D1, 6/26, and W83

Bacterial strain	MIC (μg/ml)				
	AZM	ERY	AMP	OFX	GEN
P. gingivalis 381	0.5	0.5	0.08	0.5	>640
P. gingivalis HW24D1	0.3	0.16	2.5	0.6	320
P. gingivalis 6/26	5	1.25	0.08	1.25	10
P. gingivalis W83	0.6	0.16	640	0.3	640

RESULTS

MICs for *P. gingivalis* planktonic cells. MICs of AZM, ERY, AMP, OFX, and GEN for *P. gingivalis* strain 381, HW24D1, 6/26, and W83 are shown in Table 1. MICs for AZM, ERY, and OFX were similar for each strain examined; however, AMP MICs showed large differences among the strains. GEN was ineffective against the biofilms of the four strains examined.

Susceptibilities for *P. gingivalis* adherent cells. We measured susceptibilities of antibiotics using a static model and established the MBICs. The MBICs of AZM, ERY, AMP, OFX, and GEN against four strains of *P. gingivalis* are summarized in Table 2. In *P. gingivalis* strain 381, the OD of the adherent cells was significantly decreased at AZM concentrations of ≥0.125 μg/ml compared with that of the control group. In *P. gingivalis* HW24D1, 6/26, and W83, the OD of the adherent cells was significantly decreased at AZM concentrations of ≥0.00061, ≥1.25, and ≥0.3 μg/ml, respectively. AZM inhibited *P. gingivalis* adherent cells of all the strains at sub-MIC. ERY decreased adherent cells of *P. gingivalis* 381, HW24D1,

TABLE 2. MBICs of AZM, ERY, AMP, OFX, and GEN for adherent cells of *P. gingivalis* strains 381, HW24D1, 6/26, and W83 using a static model

Bacterial strain	MBIC (μg/ml)[a]				
	AZM	ERY	AMP	OFX	GEN
P. gingivalis 381	0.125	0.25	20	1.25	>640
P. gingivalis HW24D1	0.00061	0.00061	>640	2.5	>640
P. gingivalis 6/26	1.25	0.6	160	640	640
P. gingivalis W83	0.3	0.16	160	1.25	>640

[a] Antibiotics could inhibit biofilm formation in a static model at sub-MICs.

6/26, and W83 at concentrations of ≥0.25, ≥0.00061, ≥0.6, and ≥ 0.16, respectively. This meant that adherent cells of three strains except for W83 were significantly inhibited at their sub-MIC levels compared with the corresponding control groups. In the AMP-treated group, the MBICs of strains 381, HW24D1, and 6/26 were higher than their MICs. In strain W83, MBIC of AMP was below its MIC; however, the MIC was much higher than the breakpoint of AMP for anaerobic bacteria (0.25 μg/ml) (6). In the OFX-treated group, the MBICs for all the strains tested were above their MICs. The MBICs of GEN were ≥640 μg/ml in tested strains, and they showed no inhibition of biofilm elimination.

Susceptibilities for *P. gingivalis* biofilms. In *P. gingivalis* 381, HW24D1, 6/26, and W83, the ATP values of biofilm cells from the flow cell biofilm model were significantly decreased by AZM at all concentrations (0.125, 1, and 10 μg/ml) compared with the control group (Fig. 1A, C, E, and G). In the *P. gingivalis* 381 and W83 groups, the ATP values were significantly decreased at ERY concentrations above their MICs (Fig. 1B and H), whereas strains HW24D1 and 6/26 did not show an inhibitory effect at their sub-MICs (Fig. 1D and F).

CLSM observations. In the control group of every strain, biofilms observed consisted mostly of live cells, and their thickness was 20 to 60 μm. However, biofilms treated with sub-MIC AZM (0.125 μg/ml) were decreased in thickness and density. In groups treated with 1 and 10 μg/ml AZM, live cells in the biofilms almost disappeared (Fig. 2). In *P. gingivalis* strain 381, ERY treatment decreased the density of the live cells in the biofilm at a concentration of 1 and 10 μg/ml, which was above the MIC (data not shown).

Drug susceptibility for biofilm-detached cells exposed to sub-MIC levels of AZM. MICs for AZM and ERY for biofilm-detached cells exposed/unexposed to sub-MICs of AZM are shown in Table 3. MICs for *P. gingivalis* exposed to AZM were not increased compared with those for unexposed cells, and exposure to sub-MICs of AZM did not cause resistance to AZM. Similarly, MICs for ERY for biofilm-detached cells exposed to sub-MICs of AZM were not above the MICs for unexposed cells, and resistance to ERY was not observed.

DISCUSSION

The macrolide antibiotic AZM is effective against chronic infections caused by *P. aeruginosa* biofilms, such as cystic fibrosis or DPB (27, 42); however, it has not been shown to have efficacy against oral biofilms of many bacterial species. AZM is already in clinical use, and there are many reports about its efficacy both *in vitro* and *in vivo* (9–11, 18, 38). However, the mechanism of its effects has yet to be determined. Oral biofilms consist of many bacterial species (19), and their structure and biological properties differ in each microenvironment. It is difficult to assess the mechanism of action of antibiotics and their effects against oral biofilms that comprise mixed species *in vitro*. *P. gingivalis*, which is one of the key pathogens in oral biofilm infections, was selected for the present study to clarify the effect of AZM on *P. gingivalis* biofilms using ATP measurement and CLSM *in vitro*. Mono- and multispecies biofilms differ with regard to structure, and therefore, it might change the permeability to antibiotics and exert an influence on their effects. The extracellular polymeric substances and biofilm-forming bacteria differ from those in actual oral biofilms; thus, it will be necessary to verify the results *in vivo*.

The genotype of *fimA*, which encodes an important fimbrial protein, FimA, is classified into six groups, from I to V and Ib, because of slight differences in nucleotide sequence and is related to expression of pathogenicity of *P. gingivalis* (28). A lot of research has been carried out to study the relationship between the stage of progression of periodontitis and differences in genotypes (2, 24, 35, 41). These studies have shown that, in severe marginal periodontitis, type II is most frequently detected, followed by type IV (2, 24). Similarly, in apical periodontitis, type II *P. gingivalis* shows the highest appearance ratio in symptomatic focus and type I is isolated from asymptomatic focus (41). The present study was designed to investigate the effect of antibiotics on adherent cells and multilayer biofilms. AZM inhibited biofilms of all the *P. gingivalis* strains tested in the static and flow cell models at sub-MICs (Table 2, Fig. 1). *P. gingivalis* biofilms decreased in thickness after the addition of AZM, and in the presence of 1 and 10 μg/ml AZM, bacterial cells in the biofilm almost disappeared (Fig. 2). Promotion of biofilm detachment or some impact, such as water flow, on the biofilm matrix is considered a possible reason. It has been reported previously that AZM and ERY are effective biofilm inhibitors in a static model (38), and this is similar to our results with biofilm adherent cells in the static model. However, in the flow cell biofilm model, ERY was ineffective for biofilm inhibition. This differs from the static model and previous studies (38). The flow cell biofilm model resulted in formation of a *P. gingivalis* biofilm with a >20-μm thickness. With regard to actual dental biofilms, the flow cell biofilm model is preferable for investigation of the antibiofilm effect.

AMP and OFX were examined for activity against all the strains in a static model, and they inhibited *P. gingivalis* biofilms at concentrations over their MICs, except for AMP inhibition of strain W83. Previous studies have shown that inhibitory concentrations against biofilms are often 10 to 1,000 times higher than MICs for planktonic cells (15, 22, 25, 30). In the present study, a similar tendency was seen for AMP and OFX. GEN showed no inhibitory effect for *P. gingivalis* in planktonic and adherent cells. We found that AMP, OFX, and GEN were ineffective against adherent *P. gingivalis* cells; therefore, we predicted that these antibiotics would be ineffective against *P. gingivalis* biofilm cells in the flow cell model and did not use that model. These results with AMP, OFX, and GEN led us to suggest that AMP and OFX are effective in the acute stage of biofilm infection, but they might be useless in the chronic stage. Even when single antibiotics are not effective to inhibit

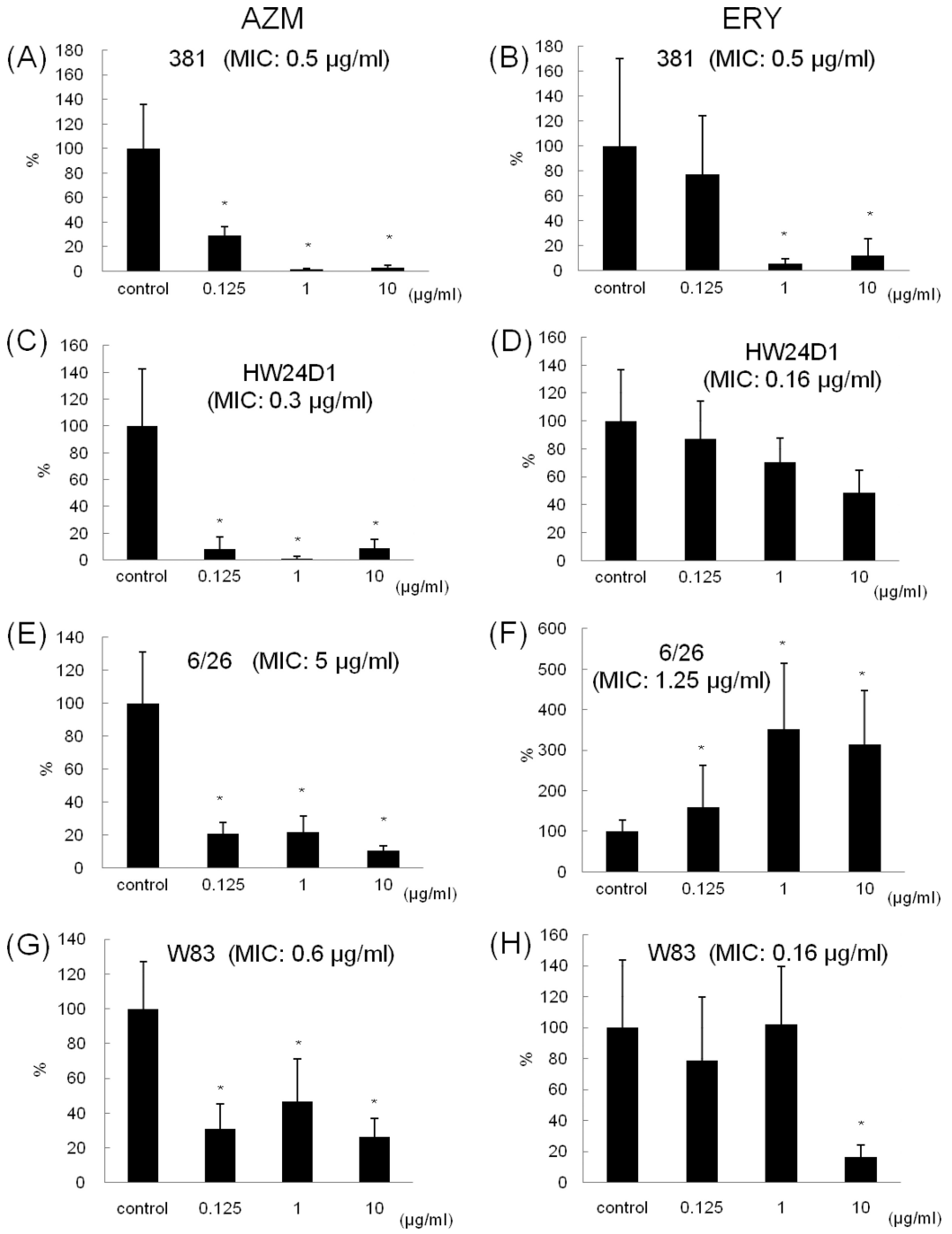

FIG. 1. Effects of antibiotics on biofilms of *P. gingivalis* strains 381, HW24D1, 6/26, and W83, determined by using the flow cell model. Biofilms of *P. gingivalis* strain 381 were prepared by using perfused culture medium and an MRD for 14 days, and then each antibiotic was added for 3 days and ATP bioluminescence assay was performed. The ATP volume of the control group was set to 100%, and these figures indicate the percentage amounts of ATP when antibiotics were added. AZM inhibited biofilms at sub-MIC levels. ERY decreased ATP levels of the biofilm cells at levels over its MIC in strains 381 and W83. (A) AZM for strain 381; (B) ERY for strain 381; (C) AZM for strain HW24D1; (D) ERY for strain HW24D1; (E) AZM for strain 6/26; (F) ERY for strain 6/26; (G) AZM for strain W83; (H) ERY for strain W83. Ten biofilm samples were examined at each concentration. *, $P < 0.005$ (Student's t test).

biofilms significantly, we can strengthen the antibiofilm effect by combination with other agents (5, 36). When adjunctive antibiotics are administered, it would appear that they can exhibit wider antimicrobial activity.

The acquisition of drug resistance because of sub-MICs of antibiotics has been a problem. Among children, AZM-resistant bacteria are detected in 85% of patients after 6 weeks of oral administration, and AZM shows more resistance than

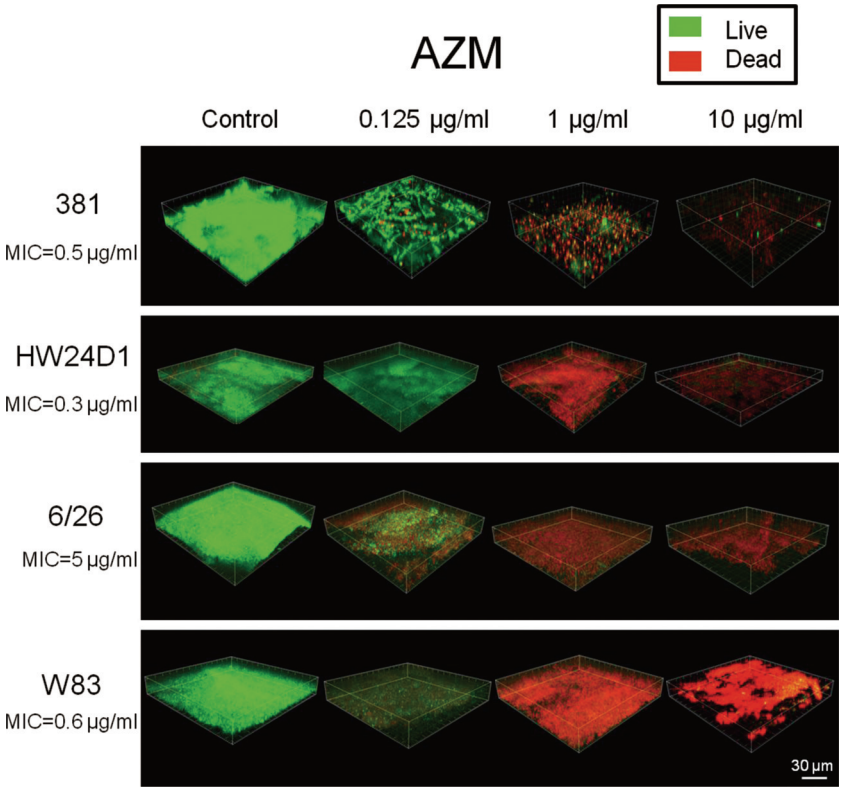

FIG. 2. CLSM images of AZM-treated biofilms of *P. gingivalis* strains 381, HW24D1, 6/26, and W83. *P. gingivalis* biofilms were formed on celluloid disks, and after adding AZM, biofilms were stained by LIVE/DEAD staining. Biofilms were observed using CLSM. Images were processed using Imaris software. Green shows live cells and red dead cells. In all the strains, at the higher concentrations of AZM, fewer residual cells were observed.

other macrolide antibiotics do (17). Several other reports about increasing AZM resistance have been published recently (1, 12, 26). However, we did not find any evidence of acquisition of resistance after exposure to sub-MICs of AZM or ERY (Table 3). Moreover, clinical isolates of *P. gingivalis* have been reported not to show resistance to AZM (16, 40), and these data support the future possibility of clinical application of AZM as a new therapy for chronic infections caused by biofilms.

TABLE 3. Susceptibility of detached cells from biofilms of *P. gingivalis* strains 381, HW24D1, 6/26, and W83 exposed to sub-MICs of AZM[a]

Bacterial strain	MIC (μg/ml)			
	AZM		ERY	
	Unexposed	Exposed	Unexposed	Exposed
381	1.56	0.39	0.19	0.09
HW24D1	0.39	0.09	0.09	<0.02
6/26	6.25	6.25	0.78	0.78
W83	6.25	6.25	0.78	0.78

[a] Culture fluid of *P. gingivalis* was perfused for 14 days using a flow cell model, and biofilms were formed. Medium that contained a sub-MIC of AZM was perfused for 3 days, and biofilm-detached cells were collected. Controls were perfused with hemin-and-menadione-supplemented GAM broth only. MICs were determined for *P. gingivalis* biofilm-detached cells exposed/unexposed to sub-MICs of AZM, according to the standard method of the Japan Society of Chemotherapy.

In conclusion, the present study demonstrated the efficacy of AZM at subinhibitory concentrations, and it was capable of inhibiting *P. gingivalis* biofilm. We suggest that AZM is likely to be useful for the treatment of diseases caused by *P. gingivalis* biofilms. However, the mechanism of action of AZM against *P. gingivalis* biofilms has not yet been clarified and remains a subject for future studies.

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