2. Human saliva collection

Whole saliva samples were collected from 5 healthy human subjects (28—30 years old) after stimulation by chewing paraffin gum, and pooled in ice-chilled sterile bottles for 5 min. The samples were clarified by centrifugation at 10,000×*g* for 20 min at 4℃, filter-sterilized, and used immediately for biofilm assays, employing a flow cell system.

3. Biofilm formation assay using flow chamber system

Biofilm formation by all strains was assayed using a method described previously, with some modifications[28,30]. Biofilm samples were cultivated at 37℃ in 3-channel flow cells[31] with individual channel dimensions of 1×4×40 mm and supplied with a flow of tryptic soy broth without dextrose including 0.25% sucrose (TSBS). The flow cell system (The Stovall Flowcell ; Stovall Life Science Inc,. Greensboro, NC, USA) was assembled and prepared as described by Christensen *et al.*[32]. The substratum consisted of a microscope glass cover slip. The flow cells were covered with filter-sterilized human saliva samples and left for 30 min. After the saliva was removed, 0 or 1×10^7 of *S. mutans* and 0, 1, 10, or 100×10^7/mL of *E. faecium*, *L. casei*, or *L. salivarius* were inoculated together in the flow cell. Following that inoculation period, medium flow was stopped for 1 h. Next, the flow was started again and the medium was pumped through the flow cells at a constant rate of 3 mL/h for 16 h using a peristaltic pump (Ismatec ; IDEX Corp., Glattbrugg-Zürich, Switzerland).

4. Confocal laser scanning microscopy

Non-invasive confocal imaging of fully hydrated biofilm samples was performed using a Fluoview CLSM (Olympus, Tokyo, Japan), fitted with a water immersion dipping objective lens (×100) and a Kr-Ar laser[30]. The specimens were stained for 30 min with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit solution (4 mL of distilled water containing 6 μL each of components A and B, Molecular Probes, Leiden, The Netherlands). The biofilm structure was analyzed using a series of horizontal (*xy*) opt digital sections, each being 5.0 μm thick, with the intervening gaps between the horizontal sections ranging from 0 to 20 μm over the entire height (*z*-axis) of the biofilm. In addition, we analyzed vertical (*xz*) sections, which were recorded from the center of each biofilm, to determine the architecture. Each biofilm was scanned at 5 randomly selected positions away from the disk edge. The digital images were processed using Fluoview software version 2.0 (Olympus, Tokyo, Japan).

5. SE preparation

E. faecium was obtained by centrifugation (10,000×*g*), and then washed 3 times with phosphate-buffered saline (PBS, pH 7.2). Sonic extracts (SEs) from the harvested bacterial cells were prepared by a method reported previously[33]. Briefly, the bacterial cells were re-suspended in PBS and sonicated 40 times for 30 s on ice with a sonicator (Sonifier 250D, Branson Ultrasonics. Co., Danbury, CT, USA). Disruption of the bacteria was confirmed by microscopy. The samples were centrifuged at 8,000×*g* for 30 min and cell-free supernatants were stored at −20℃ until use in the experiments. Protein concentrations in the SEs were estimated using a BioRad protein assay kit (Bio-Rad, Richmond, CA, USA).

6. Gel filtration

Proteins in the SE samples were precipitated by salting out, utilizing 60% ammonium sulfate. The precipitates were suspended and dialyzed in 20-mM Tris buffer (pH 7.4). The prepared SE sample was then applied to a Sepharose 4B column of 26×100 cm (Amersham Pharmacia Biotech, Buckinghamshire, UK) pre-equilibrated with the same buffer. Nine-mL fractions were collected, then monitored for UV absorbance and inhibitory effects on biofilm formation by *S. mutans* MT8148. Active fractions were pooled and the proteins were again precipitated by salting out with ammonium sulfate. The precipitates were collected by centrifugation at 10,000×*g*, then suspended and dialyzed in PBS (pH 7.4). Protein concentrations in the sample solution were determined using a protein assay kit (BioRad). The samples were also used in other experiments.

Results

1. Effects of *E. faecium* on *S. mutans* biofilm formation in flow cell system

Significant *S. mutans* biofilm formation was shown in both the flow cell system and CLSM images (Fig. 1A and B), whereas none was observed with *E. faecium* (Fig. 1A), *L. casei* (Fig. 2B), and *L. salivarius* (data not shown) mono-cultures. Optical sections 5 μm apart and showing *xy* planes were analyzed in CLSM images of mixed biofilms formed by *S. mutans* and *E. faecium*. In 5 sections (0 to 20 μm along the *z* axis), the biofilm volumes in the CLSM images were assessed, as shown in Fig. 1. *E. faecium* showed cell number-dependent inhibition of biofilm formation on co-culture with *S. mutans* (Fig. 1A). Irregular biofilms with increased numbers of voids and hollows were observed in cultures of *E. faecium* and *S. mutans* at ratios of 100 : 1 and 10 : 1, as compared to the biofilm of mono-cultured *S. mutans* (Fig. 1B). The diameters of circular voids in each section were measured at the base (0 μm) of 6 sites selected randomly in the mixed and single biofilm samples, and found to be significantly larger in the mixed culture biofilms of *E. faecium* and *S. mutans* at ratios of 100 : 1 and 10 : 1 (28.9 ± 4.0 and 20.3 ± 1.9 μm, respectively), as compared to those of the *S. mutans* mono-cultured biofilm (14.4 ± 2.4 μm). However, increasing the ratio of *L. casei* or *L. salivarius* with *S. mutans* did not significantly affect biofilm formation (Fig. 2A, B).

2. Inhibitory effects of SE sample

To confirm the inhibitory activities of *E. faecium*, an SE sample was extracted. The SE sample showed a significant inhibitory activity against biofilm formation of *S. mutans* MT8148 in the 96-well microtiter plate assay (unpublished data). Moreover, to clearly observe the inhibitory effects, the sample was sub-purified from its SE by salting out and gel filtration. A 4-fold purification of the inhibitory substance was achieved, and a high molecular weight complex>600 kDa was observed by SDS and native-PAGE in the sub-purified SE sample (unpublished data). The sub-purified sample was further applied to the biofilm formation assay using the flow cell. Applications of 25 and 50 μg/mL of sub-purified SE samples inhibited biofilm formation by *S. mutans* MT8148 in comparison with 0 μg/mL, and the level of inhibition was greater with the higher concentration in the flow cell system (Fig. 3). Morphological inhibition by each sub-purified SE sample shown was similar to that in biofilm with a larger void induced by the *E. faecium* cells in CLSM images.

Discussion

Oral streptococci have a strong tendency to colonize tooth surfaces, and the subsequent bacterial multiplication, adherence of additional bacterial species, and production of extracellular polymers on colonized tooth surfaces is referred to as oral biofilm[2,5,11]. Mutans streptococci in particular are able to induce a low-pH environment and so associate with other species such as lactic acid bacteria during biofilm formation. In contrast, lactic acid bacteria may control the environment during the development of dental caries[1,2,6,7].

Under the present test conditions, mono-cultures of *E. faecium*, *L. casei*, and *L. salivarius* failed to form substantial biofilms. Further, more mixed cultures of *S. mutans* and *E. faecium* formed irregular biofilms with an increased number of voids and hollows, observed in ratios of *E. faecium* to *S. mutans* of 10 : 1 and 100 : 1. *E. faecium* did not co-aggregate with *S. mutans* or form biofilm in the present flow cell system; thus, other diverse mechanisms are likely to be involved. It is possible that *E. faecium* produces competitive factors for binding to a saliva-coated glass surface or anti-bacterial agents against *S. mutans*. In fact, our results showed that *E. faecium* produced a substance which inhibited biofilm formation by *S. mutans*. Previous studies have reported that some strains of *Enterococcus* produce cytolysin, which has hemolytic (lysing a broad spectrum of cells, including human, horse, and rabbit erythrocytes) and bactericidal activities against gram-positive bacteria[34,35]. However, the strain used in the present experiments does not produce cytolysin. Thus, it is considered that the SE sample may have included other inhibi-

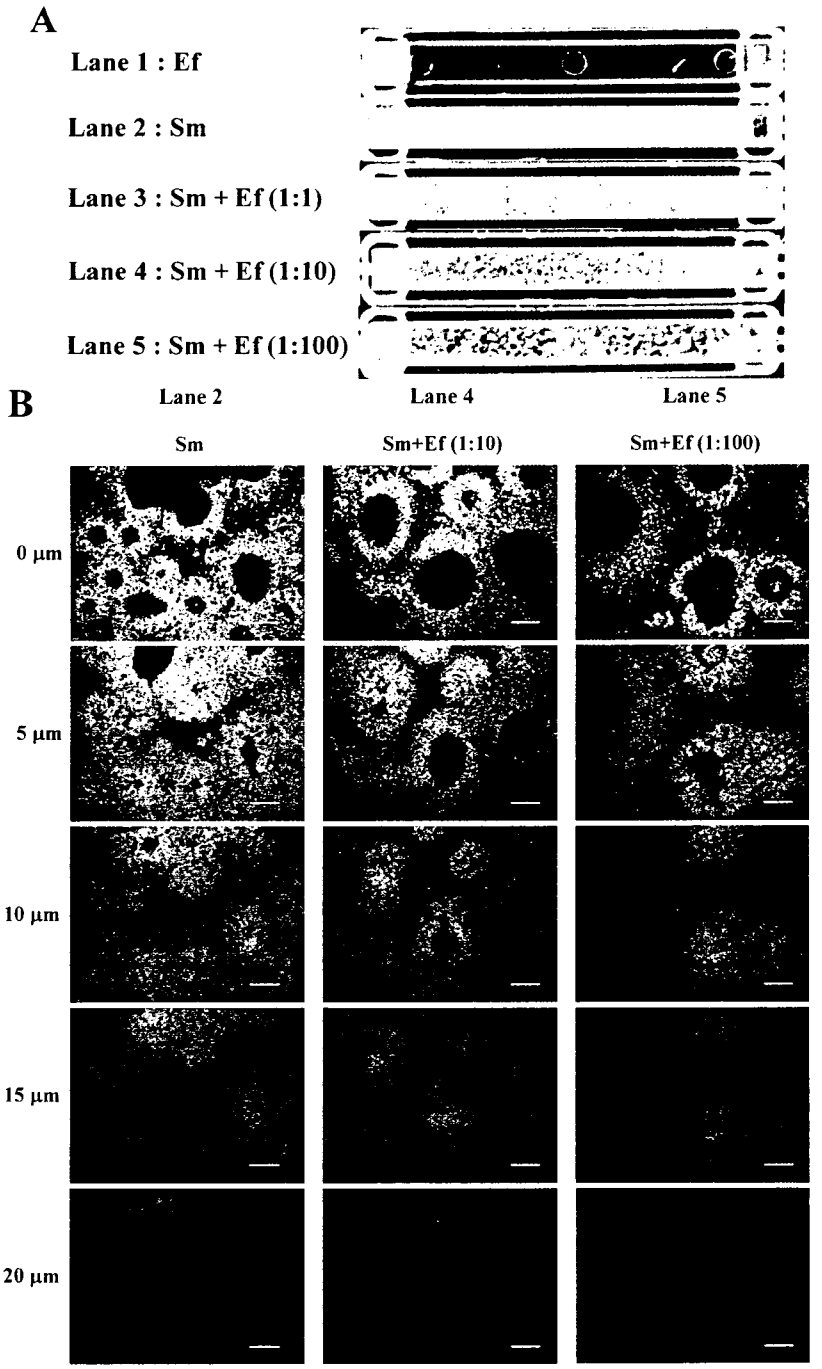

Fig. 1 Biofilm formation by *E. faecium* and *S. mutans* in a flow cell system

(A) : Biofilm formation following mono- and co-cultures of *S. mutans* (Sm) and *E. faecium* (Ef) in a flow cell culture system. Lane 1 : Ef (5×10^6 CFU/lane), Lane 2 : Sm (5×10^6 CFU/lane), Lane 3 : Sm (5×10^6 CFU/lane) + Ef (5×10^6 CFU/lane), Lane 4 : Sm (5×10^6 CFU/lane) + Ef (5×10^7 CFU/lane), Lane 5 : Sm (5×10^6 CFU/lane) + Ef (5×10^8 CFU/lane). The images shown are representative of 5 independent experiments, with similar results obtained in each. (B) : CLSM *xy* images of biofilms formed by mono- and co-cultures of *S. mutans* (Sm) and *E. faecium* (Ef). Live (green) and dead (red) cells in the biofilm were stained with SYTO9 and propidium iodide, respectively. Lanes 2, 4, and 5 in Fig. 1A are presented. Marks from 0—20 μm show the distance from the bottom of the biofilm. Bars = 20 μm. Images shown are representative of 5 independent experiments, with similar results obtained in each.

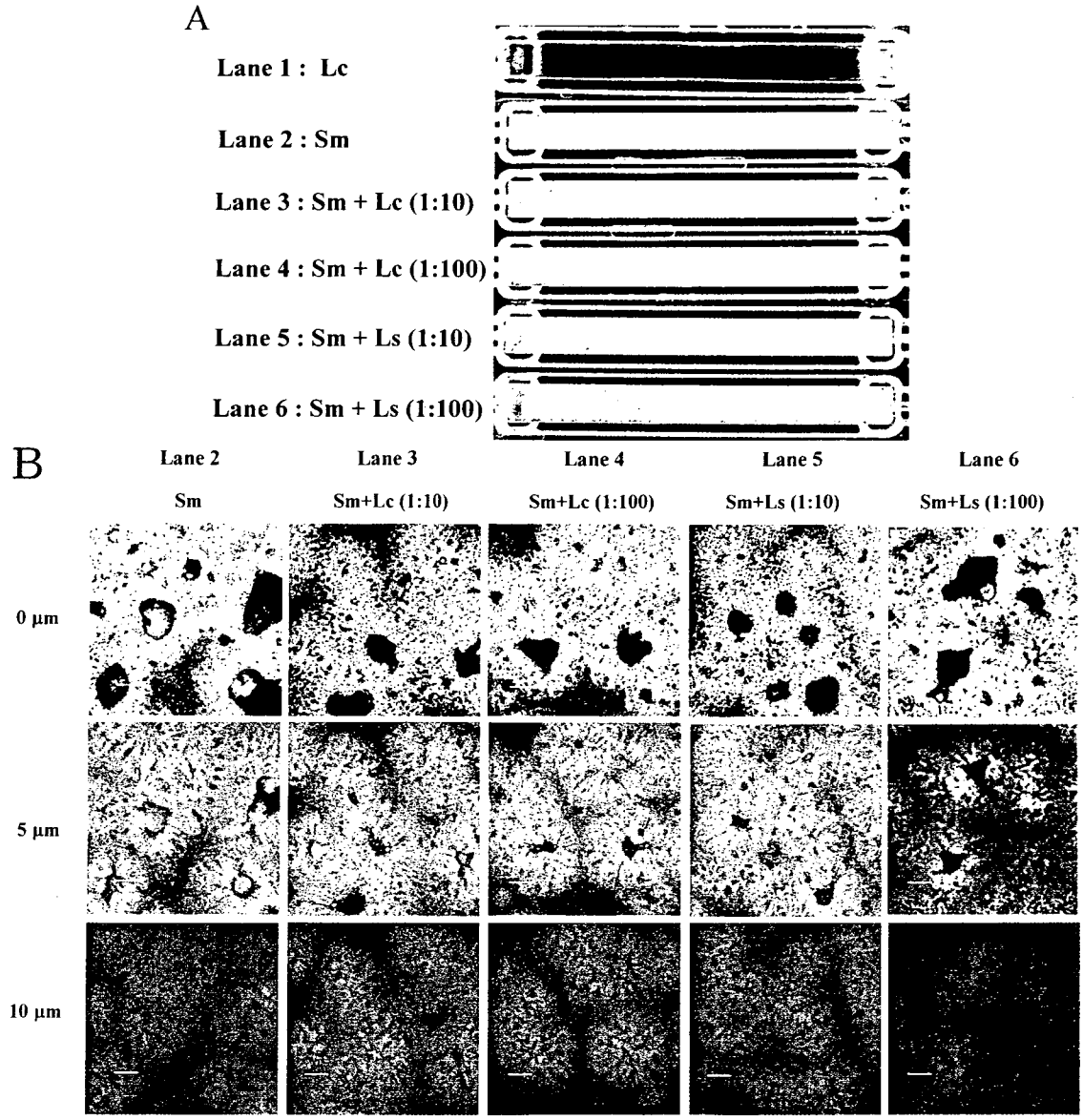

Fig. 2 Biofilm formation by *Lactobacillus* and *S. mutans* in a flow cell system
(A) : Biofilm formation with mono- and co-cultures of *S. mutans* (Sm) and *L. casei* (Lc) or *L. salivarius* (Ls) in a flow cell culture system. Lane 1 : Lc (5×10^6 CFU/lane), Lane 2 : Sm (5×10^6 CFU/lane), Lane 3 : Sm (5×10^6 CFU/lane) + (5×10^7 CFU/lane), Lane 4 : Sm (5×10^6 CFU/lane) + Lc (5×10^8 CFU/lane), Lane 5 : Sm (5×10^6 CFU/lane) + Ls (5×10^7 CFU/lane), Lane 6 : Sm (5×10^6 CFU/lane) + Ls (5×10^8 CFU/lane). Images shown are representative of 3 independent experiments, with similar results obtained in each. (B) : CLSM *xy* images of biofilms formed by mono- and co-cultures of *S. mutans* and Lactobacilli. Dead (red) and live (green) cells in the biofilm were stained with propidium iodide and SYTO9, respectively. Lanes 2, 3, 4, 5, and 6 in Fig. 2A are presented. Marks from 0—10 μm show the distance from the bottom of the biofilm. Bars = 20 μm. Images shown are representative of 3 independent experiments, with similar results obtained in each.

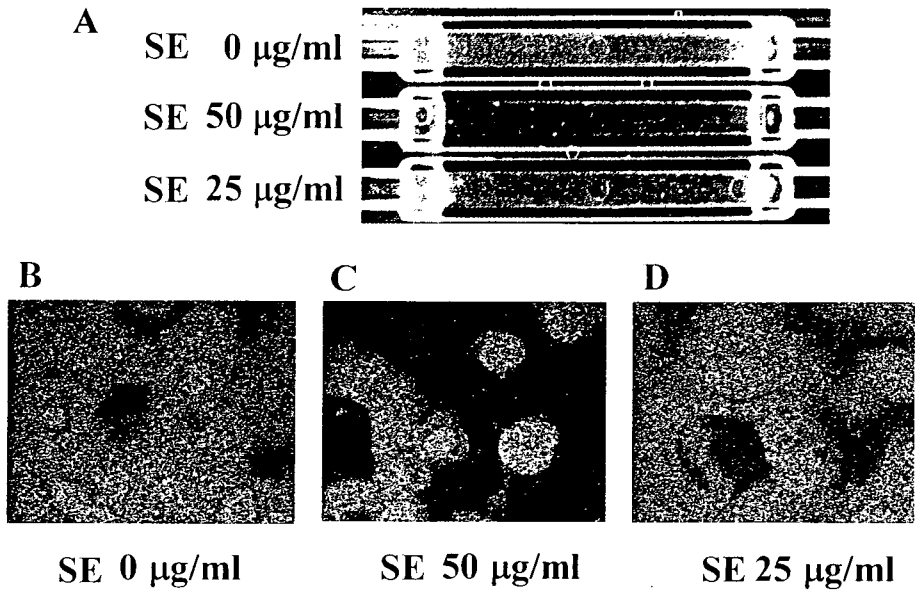

Fig. 3 Inhibitory effects of HMWC on *S. mutans* biofilm formation in a flow cell system

(A) : Photographs of biofilm formation by *S. mutans* MT8148 with 0, 25, and 50 μg/mL of HMWC in a flow cell culture system (actual size). (B), (C), (D) : CLSM *xy* images of biofilms formed by *S. mutans* cultured with 0, 50 and 25 μg/mL, respectively, of sub-purified SE sample. The images shown are representative of 3 independent experiments, with similar results obtained in each.

tors and reacted with biofilm-producing bacteria to reduce attachment of the organisms to the glass surface as well as colonization, leading to a poor quality biofilm with an increased number of voids and hollows (Figs. 1, 2). Culture supernatant from *E. faecium* did not show a significant inhibition of *S. mutans* biofilm formation. Therefore, these inhibitory effects of *E. faecium* may require cell density-dependent bacterial contact and cell lysis, and the binding of products to a salivary receptor of biofilm bacteria on a hard surface during biofilm development.

In the present study, the lactic acid bacteria *L. casei* and *L. salivarius* did not show abilities to produce biofilm in mono-cultures, nor did they inhibit biofilm formation or the attachment of *S. mutans* in co-cultures with increasing ratios. In general, lactobacilli are major odonto-pathogens[36]. Filoche *et al*[37]. reported that *S. mutans* markedly promoted the growth of lactobacilli-based biofilm, and suggested that the ability of individual bacteria to form a mono-culture biofilm was not necessarily an indicator of their survival or pathogenic potential in a complex multi-species biofilm community. However, soluble extracts of *L. fermentum* were previously shown to be able to inhibit the growth of *S. mutans* and formation of insoluble glucan produced by *S. mutans*[15,16]. *Lactobacillus rhamnosus* GG, another well-known probiotic strain, was also recently reported to be able to promote oral health in children and adults[38]. Further, more enhanced biofilm growth was observed in experiments with *Lactobacillus plantarum*, which has been implicated in the development of dental caries[36,39].

Taken together, these reports and results show that lactic acid bacteria including lactobacilli and *E. faecium* play multiple roles as unique modulators of both pathogenic and normal microflora on tooth surfaces. Additional investigations are required to clarify whether the products inhibit colonization or attachment in a physical or chemical manner during *S. mutans* biofilm formation on a hard surface, as well as to purify the tested products, before a definitive conclusion regarding the mechanism of early-onset inhi-

bition can be presented. Nevertheless, the present results should provide useful information to help understand oral biofilm formation, as well as for the development of therapies and materials for the prevention of biofilm development in the oral cavity.

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歯科医療機関における院内感染対策の導入について

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はじめに

歯科医療における院内感染対策についての重要性が認識されてきたせいか，現在それに関する雑誌や書籍等が刊行されている[1,2]．歯科医師会や自治体が主催する研修会や講習会も行われており，院内感染対策が大きな時代の流れになってきていることは間違いない．それらの内容には，洗浄や消毒，滅菌方法，院内環境の整備，情報の収集，スタッフへの教育，研修会への参加等が含まれており，そのどれを取っても，院内感染対策として有効なものであろう．しかし，現実にはマスクやグローブ，防護用メガネすべてを着用して診療している歯科医師は，関東地区で約37%（平成16年度の厚生労働科学研究班において，歯科医師会所属の歯科医師3,912人にアンケート調査を行った．その有効回答742人のうちの37%）と低いことから[2]，浸透していくにはまだまだ道のりが長い．

一方で，“経営していくのが精一杯で，そこまで手が回らない”や“今まで特に気にしなかったし，結果として問題なく歯科医療をやってこれたから，今後もこの方法でよい”と考えている歯科医師もいるだろう．気づかないうちに世の中の流れに取り残されていき，最後は患者にそっぽを向かれる歯科医院が増えたら，歯科医療全体の信頼を損ない兼ねない，と危惧する．

本稿では，歯科医療における院内感染対策を簡単にまとめ，さらに歯科医療における院内感染対策をどのように導入していけるかについて，われわれが行った歯科医師を対象としたアンケート調査の結果から考察していきたい．

Ⅰ．歯科医療における院内感染対策のまとめ

現在考えられる院内感染対策の項目について，下記にまとめた．

① スタンダードプリコーション（ユニバーサルプリコーション）の理解：すべての患者は感染症を

有しているものとみなし，患者からの血液や唾液，分泌液，滲出液，組織，抜去歯牙，鼻汁などを感染源として考え，院内感染対策の対象物として扱う．

② マスク，グローブ，防護用メガネの使用

③ 手洗い，手指の消毒：流水や石鹸を用いて手洗いをした後，塩化ベンザルコニウムや消毒用エタノールなどで手指の消毒を行う．

④ 患者ごとの滅菌ハンドピースの交換

⑤ 問診や問診票を用いた患者情報の収集：感染症などの疾患の既往歴，感染症が多発しているエリアへの渡航歴などの情報を収集する．

⑥ 器具の洗浄，滅菌，消毒方法の確立：超音波洗浄器による洗浄，オートクレーブを用いた滅菌，次亜塩素酸ナトリウムやグルタールなどの薬液を用いた消毒など．

⑦ 印象材の消毒：一般の消毒剤を使用する．

⑧ 器具の整頓

⑨ 院内環境の清掃

⑩ 口腔バキュームや空気清浄機の設置

⑪ 医療および感染性廃棄物の処理

⑫ 針刺し事故防止対策

⑬ スタッフへの教育

⑭ 自院における院内感染対策マニュアルの作成

⑮ 研修会，講習会への参加

⑯ 外部の感染症情報の収集

*

それぞれの項目の詳細な内容は，参考文献[1,2]があるので参照していただきたい．

Ⅱ．歯科医師を対象にしたアンケート調査

平成16年度から発足した「歯科医療における院内感染防止システムの開発」の厚生労働科学研究班では，3年間に4回のアンケート調査を実施した（表1）．それぞれのアンケート調査では，意識，知識，行動の3つの項目に沿って質問内容を作成し，調査結果からそれぞれの質問項目の割合，大学卒業年度（年齢），男女，標榜科，患者人数とそれらの質問項目の関連性を解析した．

表1 歯科医院におけるアンケート調査

	対象（地域）	調査対象数	有効回答数	実施年
1	県歯会員（関東地区）	3,912人	742人（19%）	平成16年
2	市歯会員（関東地区）	135人	61人（45%）	平成16年
3	県歯会員（東海地区）	3,271人	2,018人（61.7%）	平成17年
4	県歯会員（関東地区）	3,873人	392人（10.1%）	平成18年

平成16年度の研究成果から，スタンダードプリコーション（ユニバーサルプリコーション）を理解している人は約10%と低く，過去に研修会を受けた人でも約17%にとどまり，受けていない人では5%に低下することが明らかとなった[3]．さらに平成17年度の研究成果では，大学卒業年度が最近であるほど，スタンダードプリコーションの理解率が高いことも明らかとなった[4]．防護用メガネ，マスク，グローブの着用や患者ごとのハンドピースの交換など院内感染対策の「行動」に関わる部分も，大学卒業年度やスタンダードプリコーションに関する理解と有意に相関していた．また，スタッフの教育や研修会への参加も，スタンダードプリコーションに関する理解やスリーウエイシリンジ循環水の微生物汚染に関する知識と有意に相関していた．これらの結果から，大学教育や卒後研修における院内感染対策の「知識」の蓄積が重要であることが示唆された．

平成18年度はさらに質問項目を絞り込み，年齢，来院患者数，標榜科のデータを加え，再度アンケート調査を行い，解析した．質問は，現在わが国で急激に感染者が増加しているHIV患者の歯科治療を例にして行った．その結果，以下に示す興味深いデータが得られた．

表2 「年齢」と院内感染対策に対する「意識」「知識」との関係 (単位：%)

		年齢				
		39歳以下	40～49歳	50～59歳	60歳以上	P
意識	自分の歯科医院でHIV患者を受け入れる	41.7	24.5	19.7	4.4	<0.001
	他の医院ならHIV患者の治療を行う	41.7	35.3	27.0	23.9	0.167
	HIV患者を拒否するのはよくないことだと思う	70.8	69.1	70.3	58.2	0.403
	HIV患者を治療すると他の患者が来なくなると思う	54.2	60.7	76.1	76.1	0.008
知識	スタンダードプリコーション（ユニバーサルプリコーション）を知っている	16.7	24.8	24.1	10.4	0.211
	血液の混じった唾液はHIVの感染源になると思う	83.3	85.8	79.3	74.6	0.259
	HAART療法を知っている	12.5	12.0	11.0	4.4	0.379

表3 「年齢」と院内感染対策に対する「行動」との関係 (単位：%)

		年齢				
		39歳以下	40～49歳	50～59歳	60歳以上	P
行動	●患者の有する感染症を知るための対応					
	問診票に記載してもらう	87.5	80.3	71.9	47.8	0.000
	問診で聴取する	41.7	50	46.6	55.4	0.551
	微生物検査を行う	8.3	5.6	2.7	0	0.121
	特に何もしない	0	0.7	3.4	15.4	0.000
	●治療を行うときの感染対策					
	防護用メガネを着用する	45.5	38.2	41.2	23.4	0.078
	マスクを着用する	100	97.8	97.1	92.3	
	グローブを着用する	81.8	80.1	58.7	39.1	0.000
	患者ごとにハンドピースを交換する	27.3	27.9	26.8	9.2	0.000
	スタッフに感染防止の教育を行う	95.5	83.0	76.8	64.5	0.006
	自院で感染防止マニュアルを作成する	13.6	28.1	21.2	7.9	0.010
	研修会に参加する	31.8	50.3	60.3	42.9	0.016
	HBVワクチンを接種したことがある	95.5	80.7	76.8	60.0	0.001
	スタッフはHBVワクチンを接種している	54.5	36.6	36.8	36.1	0.417

Ⅲ．年齢と院内感染対策との関係

関東地区の県歯科医師会所属の歯科医師3,873人に再度アンケート調査を行い，有効回答のあった392人（10.1%）のアンケート結果の分析を行った．歯科医師の年齢から「39歳以下」「40～49歳」「50～59歳」「60歳以上」の4グループに分け，それぞれの質問項目に対する回答の割合を算出した．

“HIV患者（の治療）を拒否するのはよくないことだと思う”という割合は，それぞれの年代で約60%以上と高いにもかかわらず，“自分の歯科医院でHIV患者を受け入れる”は「60歳以上」で4.4%と低く，「39歳以下」(41.7%) の約1/10であった（**表2**）．一方で，“HIV患者を治療すると他の患者が来なくなると思う”は「60歳以上」(76.1%) が「39歳以下」(54.2%) よりも高く，「60歳以上」の多くの歯科医師は，他の患者への風評を理由に“自院でHIV患者を治療したくない”と考えていることが明らかとなった．また，スタンダードプリコーションなどの「知識」の部分は，各年代において有意な差が認められなかった．

表4 「1日に来院する患者数」と院内感染対策に対する「意識」「知識」との関係 (単位：%)

		1日に来院する患者数					
		15人以下	16～25人	26～35人	36～45人	46人以上	P
意識	自分の歯科医院でHIV患者を受け入れる	19.2	14.2	25.6	23.5	37.0	0.049
	他の医院ならHIV患者の治療を行う	25	26.3	23.6	39.4	51.9	0.039
	HIV患者を拒否するのはよくないことだと思う	64.1	66.4	66.7	71.4	88.5	0.202
	HIV患者を治療すると他の患者が来なくなると思う	70.1	70	67.9	65.7	70.3	0.987
知識	スタンダードプリコーション（ユニバーサルプリコーション）を知っている	20.3	19.1	16.4	32.3	29.6	0.365
	血液の混じった唾液はHIVの感染源になると思う	76.9	85	83.3	74.3	81.5	0.461
	HAART療法を知っている	10.1	8.5	7.6	8.6	14.8	0.833

表5 「1日に来院する患者数」と院内感染対策に対する「行動」との関係 (単位：%)

		1日に来院する患者数					
		15人以下	16～25人	26～35人	36～45人	46人以上	P
行動	●患者の有する感染症を知るための対応						
	問診票に記載してもらう	33.8	71.6	77.2	85.2	85.2	0.001
	問診で聴取する	61.5	47.5	57.0	37.1	37.0	0.039
	微生物検査を行う	1.3	3.5	5.1	2.9	3.7	0.771
	特に何もしない	9.0	5.0	0	2.9	0	0.050
	●治療を行うときの感染対策						
	防護用メガネを着用する	18.2	35.5	39.2	45.7	55.6	0.091
	マスクを着用する						
	グローブを着用する	55.1	59.4	74.7	80.0	74.1	0.011
	患者ごとにハンドピースを交換する	24.1	22.4	20.3	37.1	39.6	0.336
	スタッフに感染防止の教育を行う	67.9	77.9	81.0	85.7	96.3	0.020
	自院で感染防止マニュアルを作成する	19.7	13.3	22.8	28.6	48.1	0.001
	研修会に参加する	48.1	52.4	50.0	60.0	55.6	0.799
	HBVワクチンを接種したことがある	68.3	77.6	78.2	82.9	85.2	0.278
	スタッフはHBVワクチンを接種している						0.000

患者の有する感染症を知るため，患者に“問診票に記載してもらう”割合は，「60歳以上」(44.8%)が「60歳未満」(70%以上）よりも有意に低く，逆に“特に何もしない”と答えた歯科医師は，「60歳以上」が15.4%と他のグループ（4%以下）よりも飛び抜けて高い割合となった（表3）．グローブの着用，ハンドピースの交換，スタッフへの教育，感染防止マニュアルの作成など「行動」に関わる部分でも「60歳以上」と「60歳未満」で大きな差が認められ，いずれも「60歳以上」で低率を示していた．

これらの調査データより，「60歳以上」の歯科医師は，院内感染に対する「意識」と「行動」が他の年代よりも大きく欠けていることが示唆された．また，HIV患者の受け入れ意識は「39歳以下」の歯科医師で高く，院内感染対策の行動にも反映されていることが示唆された．

Ⅳ．1日に来院する患者数と院内感染対策との関係

1日に来院する患者数から，「15人以下」「16～25人」「26～35人」「36～45人」「46人以上」の5グル

表6 「口腔外科の標榜」と院内感染対策に対する「意識」「知識」との関係 (単位：%)

		口腔外科の標榜		
		有	無	P
意識	自分の歯科医院でHIV患者を受け入れる	37.5	16.9	<0.001
	他の医院ならHIV患者の治療を行う	45.2	27.7	0.006
	HIV患者を拒否するのはよくないことだと思う	82.5	64.7	0.003
	HIV患者を治療すると他の患者が来なくなると思う	70.3	68.3	0.443
知識	スタンダードプリコーション（ユニバーサルプリコーション）を知っている	31.7	19.0	0.073
	血液の混じった唾液はHIVの感染源になると思う	82.3	81.2	0.504
	HAART療法を知っている	19.0	8.2	0.012

表7 「口腔外科の標榜」と院内感染対策に対する「行動」との関係 (単位：%)

		口腔外科の標榜		
		有	無	P
行動	●患者の有する感染症を知るための対応			
	問診票に記載してもらう	74.6	70.8	0.328
	問診で聴取する	66.7	47.6	0.004
	微生物検査を行う	9.5	2.9	0.025
	特に何もしない	0	5.1	0.051
	●治療を行うときの感染対策			
	防護用メガネを着用する	47.5	36.0	0.046
	マスクを着用する	100	96.0	
	グローブを着用する	80.3	61.9	0.004
	患者ごとにハンドピースを交換する	34.4	22.3	0.035
	スタッフに感染防止の教育を行う	90.0	75.8	0.008
	自院で感染防止マニュアルを作成する	32.8	18.2	0.011
	研修会に参加する	58.3	51.0	0.186
	HBVワクチンを接種したことがある	78.3	76.0	0.698
	スタッフはHBVワクチンを接種している	45.0	36.2	0.129

ープに分け，それぞれの質問項目に対する回答の割合を算出した．

“自分の歯科医院でHIV患者を受け入れる”“他の医院ならHIV患者の治療を行う”とする割合は，来院患者の増加とともに高くなる傾向が認められた（**表4**）．“HIV患者を拒否するのはよくないことだと思う”（64～89%）や“HIV患者を治療すると他の患者が来なくなると思う”（65～70%）は，来院患者数の違いに影響を受けなかった．来院患者数が多いということは忙しい反面，収入も多いことが考えられる．一方で，来院数の違いが「知識」の部分に影響を与えることはなかった．

“問診票に記載してもらう”ことで患者の感染情報を得ているのは，「15人以下」で33.8%（他のグループは70%以上）と極端に低く，反対に“問診で聴取する”は「15人以下」で61.5%と他のグループよりも高くなっていた（**表5**）．グローブの着用，スタッフへの感染防止の教育，感染防止マニュアルの作成など，来院患者数が増加するほど有意に高くなる傾向を示した．微生物検査，患者ごとのハンドピースの交換，研修会への参加など費用のかかる項目に関しては，患者数の増加との関係性は見られな

かった.

以上の結果より，収入が多いことから経済的な余裕が生まれ，その結果として感染対策ができるようになったわけではなく，手間がかかったとしても，患者数の多い歯科医院のほうが感染対策を行う意欲が高い，と考えられる．手間を惜しむことなく感染対策に力を注ぐ歯科医院が，患者との信頼関係を生み，結果的に来院患者数の増加につながっているのではないだろうか．現に，「15人以下」の歯科医院では，“問診票に記載してもらう”よりも手間のかからない“問診で聴取する”のほうが多く，また“特に何もしない”割合が他のグループより高かったことからも推察できる．

V．標榜科と院内感染対策との関係

標榜する診療科名（「一般歯科」「矯正歯科」「口腔外科」「小児歯科」）の4グループに分け，それぞれの質問項目に対する回答の割合を算出した．

「口腔外科」を標榜している歯科医師で，“自分の歯科医院でHIV患者を受け入れる”“他の医院ならHIV患者の治療を行う”“HIV患者を拒否するのはよくないことだと思う”とする割合が，標榜していない歯科医師よりも有意に高いことが明らかとなった（**表6**）．また，スタンダードプリコーションやHIVの治療方法であるHAART療法などの知識を有する歯科医師の割合も高く，「行動」に関わる多くの質問項目においても有意に高い率を示していた（**表7**）．一方，他の標榜科ではこのような差は出なかった．

これらは，大学や病院において専門的に口腔外科を学んだ歯科医師は院内感染対策に対する「意識」や「知識」が高く，「行動」にも反映されているため，と考えられる．

Ⅵ．アンケート調査のまとめ

院内感染対策を歯科医療に導入していくためには，歯科医院の経営を充実することと同じように，意欲をもつことが大事ではないだろうか．卒業後の早い時期から，積極的に口腔外科的な治療を行うような専門性を高めていき，手間を惜しまずに院内感染対策を導入していくことが，評判の良い歯科医院に発展させていくことにつながる，と考える．患者は，院内感染予防に対する歯科医院の姿勢を見て，来院行動につなげているのかもしれない．

おわりに

今回は，関東や東海地区のように院内感染に対して比較的意識の高い地域から得られたアンケート調査結果を混じえながら解説した．アンケート調査にご協力いただいた歯科医院や歯科医師会の方々に深く感謝の意を述べたいと思う．これらの論文や研究成果から，院内感染予防対策について意識が今後さらに高くなり，歯科医療の発展につながっていくことを期待する．

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ORIGINAL ARTICLE

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Treatment of *Pseudomonas aeruginosa* biofilms with a combination of fluoroquinolones and fosfomycin in a rat urinary tract infection model

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Abstract Foreign body-associated infectious disease is currently one of the most problematic hospital-acquired infections. Patients with placement of urinary catheters are especially susceptible to such infection, that is biofilm infection. In this study, we focused on the therapeutic efficacy of prulifloxacin (PUFX) against *Pseudomonas aeruginosa* OP 14-210, isolated from a patient with complicated urinary tract infection. This microbe formed a biofilm on the surface of a polyethylene tube (PT) placed in a rat bladder without surgical manipulation. In addition, we attempted to eradicate the biofilm by treatment with a combination of PUFX and fosfomycin (FOM). A single oral administration of PUFX at a dose of 20 mg/kg was effective against *P. aeruginosa* as a biofilm, yielding a significant reduction in CFU per PT of approximately 1 $\log_{10}$ CFU/PT compared with that in untreated controls. A similar therapeutic effect was also observed in levofloxacin-treated rats, and albeit slightly weaker, in ciprofloxacin-treated animals as well. Because 3 days' consecutive treatment with each fluoroquinolone did not further decrease the viable cell counts on the PT, we tested the efficacy of combining PUFX and FOM. These two drugs, administered once a day for 3 days, at doses of 20 and 100 mg/kg, respectively, resulted in significant decreases of viable cell counts on the PT of more than 1.5 $\log_{10}$ CFU/PT compared with PUFX alone ($P < 0.05$). As seen by scanning electron microscopy, destruction and disappearance of multilayer biofilms occurred after treatment with this drug combination. Such combination therapy with PUFX and FOM may be advantageous for treating biofilm-related infectious diseases.

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Key words Urinary tract infection · *Pseudomonas aeruginosa* · Biofilm · Prulifloxacin · Fosfomycin

Introduction

Bacteria on the surfaces of implanted medical devices can cause chronic infection, enhanced by biofilm formation.[1] Biofilm infection is indolent, although the stability of biofilm is a major factor in the persistence of many chronic infections. Catheter-associated urinary tract infection (UTI) is a representative biofilm disease. In complicated UTI, the frequency with which *Pseudomonas aeruginosa*, a major pathogen in biofilm-associated infection, is found, is rising.[2]

Fluoroquinolones are widely used for the treatment of both uncomplicated and complicated UTI, because they show broad activity against organisms isolated from the urinary tract, including *P. aeruginosa*. Because of their bactericidal activity on nongrowing *P. aeruginosa*[3] and their penetrability through extracellular polymeric substances,[4] fluoroquinolones show a killing effect on *P. aeruginosa* in mature biofilms.[5,6] However, biofilm bacteria show resistance to various host defense mechanisms and antibiotics, sometimes leading to the failure of clinical therapy.

Previously, in in vitro studies, we investigated the effects of combining ulifloxacin, the active form of prulifloxacin (PUFX), and fosfomycin (FOM), against *P. aeruginosa* OP 14-210, isolated from a patient with catheter-associated UTI, in mature biofilms.[5] However, *P. aeruginosa* biofilms formed in vitro were so rigid that high concentrations of antibiotics for an extended period, unlikely to be attainable in vivo, were needed to destroy them.

In this study using a rat UTI model, we examined the therapeutic efficacy of fluoroquinolones against *P. aeruginosa* OP 14-210 forming a biofilm on the surface of a polyethylene tube (PT) placed as a foreign body in the rat bladder. This model was established without surgical manipulation to minimize postoperative infiltration. In addition, we attempted to eradicate the biofilm by treatment with a

combination of fluoroquinolones and FOM. At the same time, we monitored the surface of the PT by scanning electron microscopy (SEM) to assess morphological changes and destruction of the biofilms.

Materials and methods

Bacteria, antimicrobial agents, and animals

P. aeruginosa OP 14-210, isolated from a patient with a catheter-associated UTI, was used throughout this study.[5,6] This strain belonged to serotype I and was not an alginate-overproducing strain. PUFX and FOM were supplied by Meiji Seika Kaisha (Tokyo, Japan). Ciprofloxacin (CPFX) and levofloxacin (LVFX) were purchased from Bayer Yakuhin (Osaka, Japan) and Daiichi Pharmaceutical (Tokyo, Japan), respectively. The minimal inhibitory concentrations (MICs) of antimicrobial agents against *P. aeruginosa* OP 14-210, determined by the macrodilution tube broth method, with a final inoculation of 5×10^5 colony-forming units (CFU)/ml were: 32 μg/ml for FOM, 0.25 μg/ml for ulifloxacin (an active metabolite of PUFX), 0.25 μg/ml for CPFX, 1 μg/ml for LVFX, 1 μg/ml for biapenem, 2 μg/ml for imipenem, 0.5 μg/ml for meropenem, and 128 μg/ml for ceftazidime.[7] Seven-week-old female Crj : CD rats were purchased from Charles River Japan (Yokohama, Japan) and used after 1-week adaptation in our laboratory. All experimental protocols and animal care were approved by the Institutional Animal Care and Use Committee of Meiji Seika Kaisha, Ltd.

Formation of biofilms on the surfaces of polyethylene tubes placed in the rat bladder

According to a previous report, a spiral-shaped polyethylene tube (PT; Intramedic polyethylene tubing PE50; Becton Dickinson, Sparks, MD, USA) was placed as a foreign body in the rat bladder, without surgery.[8] Briefly, a 1.5-cm-long PT was formed into a spiral and the shape fixed in a boiling water bath for 3 s. After straightening, the PT was placed in the bladder through the urethra, under anesthesia. Rats were given water containing 1 mg/ml of ampicillin (Nakalai Tesque, Kyoto, Japan), to prevent UTI, for 4 days after placement of the PT. *P. aeruginosa* OP 14-210 organisms, grown on trypticase-soy agar (Difco, Detroit, MI, USA) were harvested, adjusted to an absorbance of 0.3 at 580 nm, and further diluted ×10 with 0.033 M phosphate-buffered saline. Then 0.5 ml of bacterial suspension (approximately 5×10^6 CFU) was inoculated transurethrally into the bladder, using a 22-gauge needle with a blunt point under anesthesia, and the urethra was clamped for 4 h to prevent bacteria being washed away in the urine.

Therapeutic efficacy of fluoroquinolones and FOM against *P. aeruginosa* in a biofilm

Twenty-four hours after inoculation, *P. aeruginosa* biofilms had formed on the surface of the PT. Each fluoroquinolone (PUFX, CPFX, and LVFX) separately and/or FOM were then orally administered. Using four to nine rats for each group, treatment for a single day or 3 consecutive days by oral administration of each fluoroquinolone at a dose of 20 mg/kg body weight and FOM at 20, 50, or 100 mg/kg body weight, suspended in hydroxypropyl cellulose-SL, was evaluated. Co-administration of PUFX at a dose of 5, 10, or 20 mg/kg and FOM at a dose of 100 mg/kg was also evaluated. Twenty-four hours after the last administration, the PT was carefully removed from the bladder, transferred to a 1.5-ml Eppendorf tube containing 500 μl of phosphate-buffered saline, and sonicated for 2 min at 38 kHz in a water bath to disperse the bacteria. Then 100 μl of the sample was spread onto Mueller Hinton agar (MHA; Becton Dickinson) and cultured at 37°C for 20 h, and the number of colonies after incubation was quantified. The lower limit of detection was $10^{0.7}$ CFU/PT.

Morphological observations by scanning electron microscopy (SEM)

Just before the third administration of PUFX and/or FOM, the PT was removed from the bladder. The biofilms on the PT were fixed with 2.5% glutaraldehyde in phosphate-buffered saline, postfixed with 2% tannic acid and 1% OsO_4, and dehydrated with ethanol. The specimens were then dried in an HCP-II critical-point dryer (Hitachi, Tokyo, Japan), coated with platinum-palladium, and observed with a Hitachi S-570 scanning electron microscope.[6]

Statistical analysis

Differences in viable cell counts between control and drug-treated animals were analyzed, using Dunnett's, Tukey's, and Mann-Whitney *U*-test methods with the SAS (SAS Institute Japan, Tokyo, Japan). Differences were considered to be statistically significant when $P < 0.05$.

Results

Therapeutic efficacy of each antimicrobial agent separately

We examined the therapeutic efficacy of a single administration of each fluoroquinolone separately, and also FOM alone, against *P. aeruginosa* biofilms. The efficacy of PUFX was higher than that of CPFX and equal to LVFX at the same dose (Table 1; experiment 2; Fig. 1A). However, there was no further decrease in viable cell counts on the PT even when each fluoroquinolone was administered orally once a day for 3 days (Table 1; experiment 5). FOM showed no reduction in CFU per PT after either a single dose (Table 1; experiment 1; Fig. 1B) or when given for 3 consecutive days (Table 1; experiment 4).

Table 1. Therapeutic efficacy of fluoroquinolones and FOM against *P. aeruginosa* biofilms

	Log CFU/PT
A single oral administration	
Experiment 1 (n = 6)	
Control	7.49 ± 0.44
FOM 20 mg/kg	7.61 ± 0.20
FOM 50 mg/kg	7.30 ± 0.43
FOM 100 mg/kg	7.49 ± 0.60
Experiment 2 (n = 5–6)	
Control	7.88 ± 0.22
PUFX 20 mg/kg	6.15 ± 0.08
CPFX 20 mg/kg	7.15 ± 0.22
LVFX 20 mg/kg	5.17 ± 0.90
Experiment 3 (n = 8–9)	
Control	8.00 ± 0.25
PUFX 20 mg/kg	6.61 ± 0.50
PUFX 20 mg/kg + FOM 20 mg/kg	6.75 ± 0.41
PUFX 20 mg/kg+ FOM 50 mg/kg	6.52 ± 0.54
PUFX 20 mg/kg+ FOM 100 mg/kg	6.16 ± 0.69
3 days' consecutive oral administration	
Experiment 4 (n = 5–6)	
Control	7.80 ± 0.29
FOM 20 mg/kg	7.54 ± 0.53
FOM 50 mg/kg	7.17 ± 0.68
FOM 100 mg/kg	7.03 ± 0.93
Experiment 5 (n = 5–6)	
Control	7.28 ± 0.46
PUFX 20 mg/kg	6.11 ± 0.55
PUFX 20 mg/kg + FOM 100 mg/kg	4.34 ± 2.00
CPFX 20 mg/kg	6.50 ± 0.79
CPFX 20 mg/kg+ FOM 100 mg/kg	5.31 ± 1.64
LVFX 20 mg/kg	5.43 ± 0.73
LVFX 20 mg/kg+ FOM 100 mg/kg	3.07 ± 2.02
Experiment 6 (n = 4–6)	
Control	7.51 ± 0.42
PUFX 5 mg/kg	6.51 ± 0.59
PUFX 5 mg/kg + FOM 100 mg/kg	5.89 ± 1.36
PUFX 10 mg/kg	6.35 ± 0.78
PUFX 10 mg/kg+ FOM 100 mg/kg	6.13 ± 0.60
PUFX 20 mg/kg	5.89 ± 1.01
PUFX 20 mg/kg+ FOM 100 mg/kg	2.93 ± 1.44

Values are means ± SD

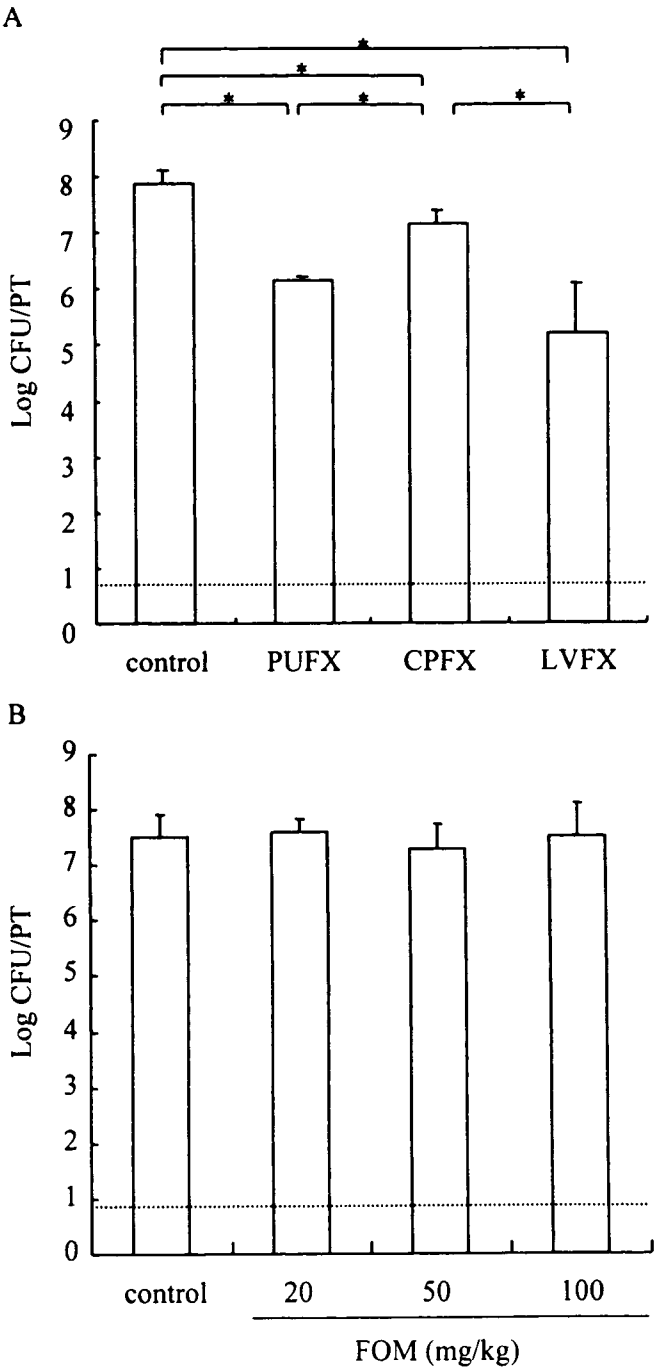

Fig. 1A,B. Therapeutic efficacy of prulifloxacin (*PUFX*), ciprofloxacin (*CPFX*), levofloxacin (*LVFX*), and fosfomycin (*FOM*) alone against *Pseudomonas aeruginosa* in a mature biofilm after a single administration. Each fluoroquinolone was administered orally to rats at a dose of 20 mg/kg body weight (**A**); FOM was administered orally at a dose of 20, 50, or 100 mg/kg (**B**). The number of viable cells on a polyethylene tube (PT) was determined 24 h after the last administration. The detection limit is indicated by the *dotted line*. Data values are expressed as means ± SD. *Statistically significant, $P < 0.05$

Treatment with combinations of fluoroquinolones and FOM

Because we observed no synergistic effects with a single co-administration of PUFX and FOM (Table 1; experiment 3), we tested whether extended treatment would achieve this. After 3 consecutive days' co-administration of PUFX and FOM, a synergistic effect was clearly identified for PUFX at a dose of 20 mg/kg plus FOM at a dose of 100 mg/kg (Table 1; experiment 6; Fig. 2). A synergistic effect was also confirmed for LVFX plus FOM and CPFX plus FOM (Table 1; experiment 5). However, we found no synergistic effects for PUFX at a dose of 5 or 10 mg/kg plus FOM at a dose of 100 mg/kg.

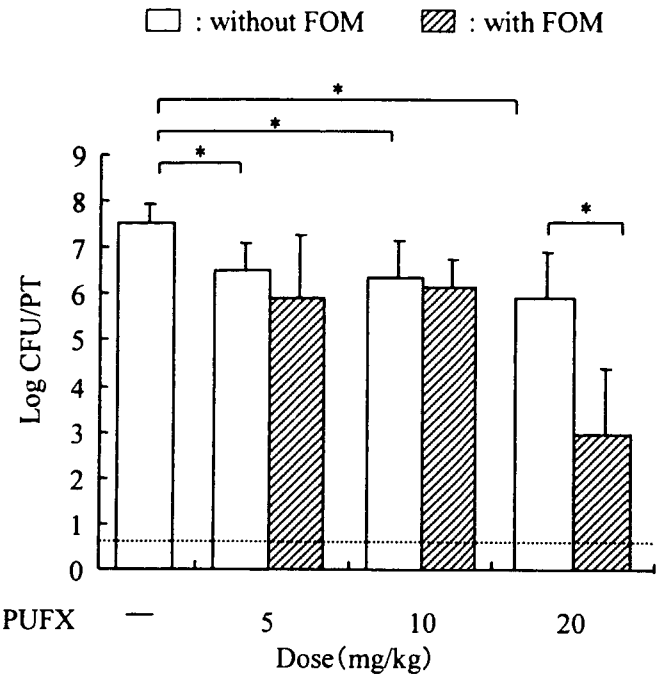

Fig. 2. Therapeutic efficacy of PUFX with or without FOM against *P. aeruginosa* in a mature biofilm after 3 consecutive days' oral administration. The number of viable cells on the PT was determined 24 h after the last administration. The detection limit is shown as the *dotted line*. Data values are expressed as means ± SD. *Open bars*, control or PUFX alone. *Hatched bars*, PUFX at a dose of 5, 10, or 20 mg/kg plus FOM at a dose of 100 mg/kg. *Statistically significant, $P < 0.05$

Morphological changes of *P. aeruginosa* on the surface of the PT after treatment with a combination of PUFX and FOM

Figure 3 shows morphological changes of *P. aeruginosa* biofilms on the surface of a PT after treatment with PUFX and/or FOM after 2 consecutive days' drug administration. The presence of rounded or elongated cells was characteristic of treatment with FOM or PUFX, respectively (Fig. 3B,C). Disappearance of multilayer biofilms, indicating their destruction, was demonstrated after combination treatment (Fig. 3D).

Discussion

In patients with long-term indwelling bladder catheterization, biofilm infections are inevitable, and the risk of infection increases by 5%–8% per day.[9] *P. aeruginosa* is a major uropathogen and is one of those most frequently isolated from urine in patients with complicated UTI. These *P. aeruginosa*-forming biofilms are well-protected from the mechanical flow of urine, host defense systems organized in the mucosal barrier, and even antimicrobial chemotherapy. The only effective method of treatment is to correct the obstruction and directly remove the biofilm. In pursuit of an efficient method for eradicating *P. aeruginosa* biofilms, we evaluated the effects of combining FOM and fluoroquinolones possessing potent antipseudomonal activity in a rat UTI model.

A previous report had indicated that a fluoroquinolone alone was unable to eradicate *P. aeruginosa* biofilms in an in vitro experiment.[5] In this rat UTI model[8] a considerable contribution from the mechanical flow of urine is relied upon to remove the biofilm. For this reason, unlike the results in an in vitro experiment, a single oral administration of each fluoroquinolone alone moderately decreased viable cell counts of *P. aeruginosa* in biofilms on the PT.

In addition to the three fluoroquinolones studied here, Neu and Chin[10] demonstrated that fleroxacin also acted synergistically with FOM against floating *P. aeruginosa* cells in an in vitro study. Other quinolones, such as gatifloxacin, moxifloxacin, sparfloxacin, and tosufloxacin play an important role in the treatment of community-acquired respiratory tract infections. These latter fluoroquinolones, however, were only equally or even slightly less potent than CPFX against *P. aeruginosa.* Considering the features of each fluoroquinolone studied here; namely, that PUFX possesses the most potent antipseudomonal activity, that CPFX can also be administered parenterally, and finally, that LVFX is the most popular fluoroquinolone, our experiments provide evidence showing synergistic effects of FOM together with fluoroquinolones for application as therapy against UTI.

Previously, Monden et al.,[11] in an in vitro study, reported that a clear decrease in the bioactivity of FOM-pretreated *P. aeruginosa* biofilms forming on silicon disks was detected as early as 24 h after treatment with FOM and ofloxacin. In our present in vivo study, a considerable concentration of FOM was maintained in the urine, mimicking the condition of pretreatment with FOM until the second administration of fluoroquinolone. Because no synergistic effects were observed with a single coadministration of PUFX and FOM, consecutive administration of fluoroquinolones and FOM would be necessary for eradicating biofilms. The feasibility of using fluoroquinolones and FOM combination therapy continuously to treat patients having catheterassociated UTI needs to be examined. However, we note that antimicrobial chemotherapy for these patients is mandatory only in the febrile phase.

Exactly how the synergistic effect of FOM with fluoroquinolones is accomplished, however, remains to be determined. The level of expression of the transport system (*sn*-glycerol 3-phoshate transport) that delivers FOM into bacterial cells is increased under anaerobic conditions,[12] but FOM did not react with a negatively charged bacterial glycocalyx.[4] These observations imply that FOM is able to penetrate deeply into multilayered biofilms and is still transported into cells in the stationary phase with a low growth rate.

In conclusion, treatment with combinations of fluoroquinolones and FOM would appear to offer improved therapy against biofilm-associated infectious diseases.

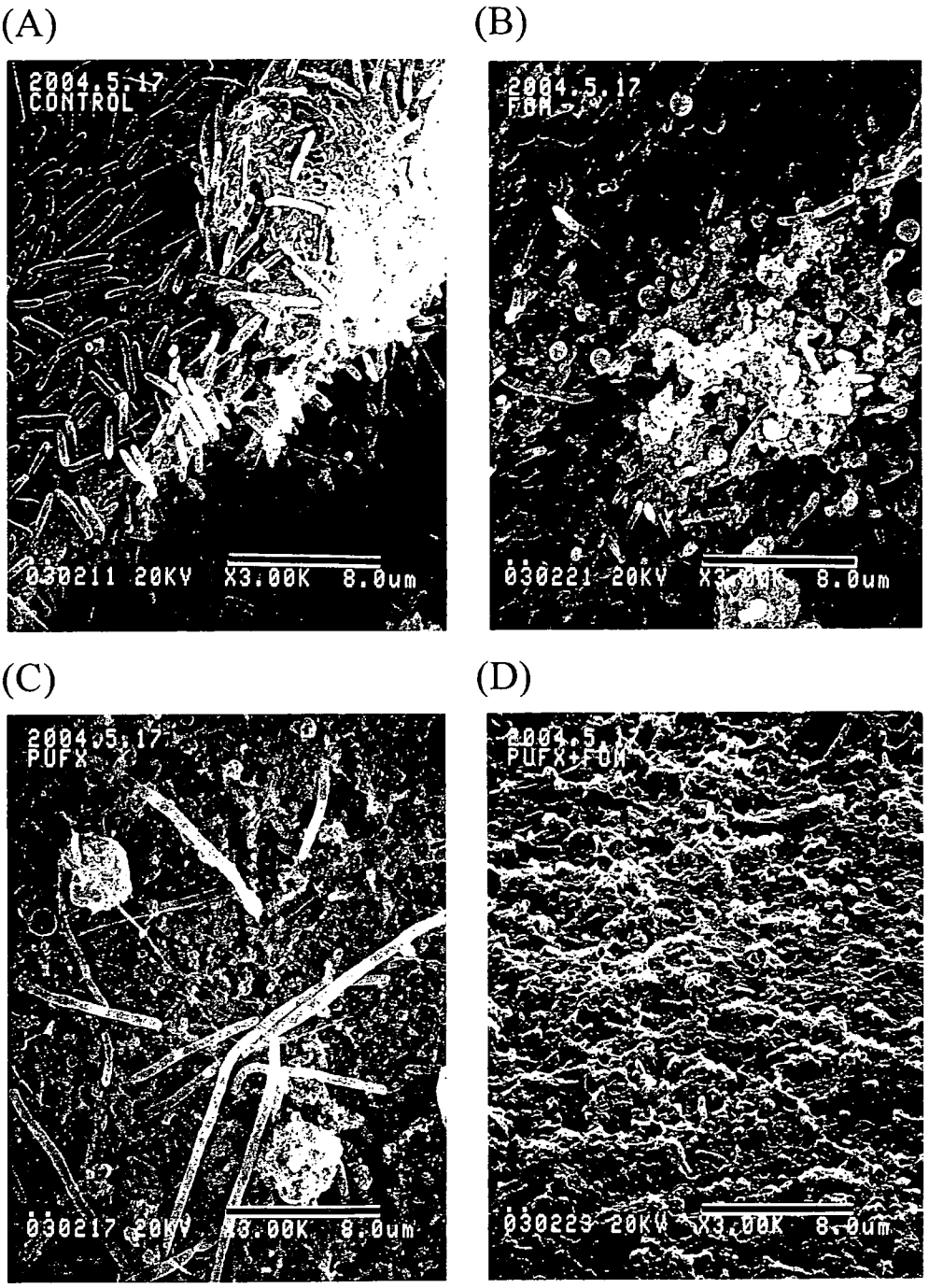

Fig. 3A–D. Morphological changes of *P. aeruginosa* in a mature biofilm on the surface of a PT placed in rat bladder just before the third administration of PUFX and FOM. **A** Untreated control, **B** FOM at a dose of 100 mg/kg, **C** PUFX at a dose of 20 mg/kg, **D** PUFX at a dose of 20 mg/kg plus FOM at a dose of 100 mg/kg. *Bars*, 8 μm

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緑膿菌性尿路感染症対策としての抗バイオフィルム剤探索とその基盤技術の開発

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I. はじめに

細菌バイオフィルムは難治性感染症に関与する病態概念として注目されており、その病態の解明や予防・治療法の開発など基礎的・臨床的研究が進展している[1-4]。尿路感染症においても、カテーテル留置感染症を代表とする難治性感染症の多くにこの細菌バイオフィルムが関与している[5-10]。複雑性尿路感染症より分離される日和見感染菌は、本来尿路への定着性は低いものの、留置カテーテルや高度の尿流障害などの基礎疾患につけこんで、細菌バイオフィルムを形成して尿路での定着性と増殖性を獲得する。尿路バイオフィルム感染症は、潜伏・持続感染の様相を呈することが多く難治性である。反復かつ遷延する尿路バイオフィルム感染症に対して、抗菌化学療法を繰り返すことによって多剤耐性菌が選択されていることも事実である。尿路バイオフィルム感染症は通常臨床症状に乏しく比較的穏やかな感染症であるが、一旦、尿流障害を合併すると尿性敗血症に移行し、宿主を重篤化させる。また、除菌が困難であるため感染が持続し、院内感染の感染源となっている。そのような背景のなかで、尿路バイオフィルム感染症の予防と制御のための新しい治療法・医用材料・抗バイオフィルム剤の開発は重要な研究課題である。

岡山大学泌尿器病態学分野では、過去10数年来、尿路でのバイオフィルム形成のモデル実験系として *in vitro*（岡山大学式ロビンスデバイス、キャピラリーフローセルシステム）および *in vivo*（ラット）の実験系を使用して、緑膿菌を中心にバイオフィルム形成と抗菌療法に関する研究を遂行してきた[5-18]。数年前に導入したキャピラリーフローセルシステム（*in vitro* 実験系）は、緑膿菌性バイオフィルム（GFP 産生株および非産生株）の観察において再現性のある実験系として進化を遂げている。本稿では、抗菌薬を含む抗バイオフィルム剤開発のためのバイオフィルム実験モデル系（キャピラリーフローセルシステム）を用いて得られた新知見を報告する。

II. 材料および方法

カテーテル留置複雑性尿路感染症患者由来の緑膿菌 *Pseudomonas aeruginosa* OP14-210 株を用いた。GFP（green fluorescent protein）産生株は、GFP をコードしたプラスミド pMF230 を OP14-210 株に導入して、*P. aeruginosa* OP14-210（pMF230）株を構築した。ガラスキャピラリー中に菌液（GFP 産生株または GFP 非産生株）を接種して、37 ℃、2 時間放置したのち、人工尿を 20 ml/hr で灌流させバイオフィルムを形成させた。人工尿における浮遊菌に対する levofloxacin（LVFX）、ulifloxacin（UFX：prulifloxacin の活性本体）、fosfomycin（FOM）の MIC は、それぞれ 8 μg/ml、2 μg/ml、64 μg/ml であった。薬剤濃度は、通常の臨床投与量で尿中に十分に到達する濃度（LVFX［80 μg/ml：10 x MIC］）、UFX［20 μg/ml：10 x MIC］、FOM［192 μg/ml：3 x MIC］）を使用した。GFP 産生株が形成した薬剤無添加と薬剤作用後のバイオフィルムを共焦点レーザー走査型顕微鏡（Zeiss LSM 510）で観察した。GFP 非産生株の場合は、蛍光染色キット（Live/Dead BacLight Bacterial Viability Kits：Molecular Probes）を用いてバイオフィルム内の生菌（Green）と死菌（Red）を染め分け、同様に観察を行った。Fig. 1 と Fig. 2 の画像解析には Imaris（Bitplane）ソフトウェアを用い、Green/Red 蛍光強度の定量化には MetaMorph（Molecular Devices）ソフトウェアを用いた。