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Bactericidal Activity in Filtrated Supernatant of *Streptococcus Sanguinis* against Multidrug-Resistant *Pseudomonas Aeruginosa*

Kiwao Watanabe,[1] Masachika Senba,[2] Akitoyo Ichinose,[3] Takeshi Yamamoto,[4] Koya Ariyoshi[1] and Keizo Matsumoto[5]

[1]Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan
[2]Department of Pathology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan
[3]Central Laboratory, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan
[4]Department of Microbiology, Kyoto University Graduate of Medicine, Kyoto, Japan
[5]Professor emeritus, Nagasaki University, Nagasaki, Japan

In the past decade, multidrug-resistant *Pseudomonas aeruginosa* (MDRP) infection has become a serious clinical problem, due to the limitation of drug choices to fight against the bacteria. Here we explored the bactericidal activity in the filtrated supernatant of *Streptococcus (S.) sanguinis* against *Pseudomonas (P.) aeruginosa*. *S. sanguinis* is one of the α-hemolytic streptococci that commonly reside in the human oral cavity. A strain of *S. sanguinis*, isolated from the sputum of a pulmonary-disease patient, was cultured for overnight. The filtered supernatant was tested for bactericidal effect using the minimum bactericidal concentration method on 20 strains of *P. aeruginosa*, including two MDRP and five mucoid-type strains. The viable number of *P. aeruginosa* was decreased with time after exposing to the filtrated supernatant of *S. sanguinis*, and collapsed bacteria were detected with electron microscopy. Of the 20 strains, 19 (95%) strains of *P. aeruginosa* were affected by bactericidal effect. Among other species of bacteria examined, the filtrated supernatant of *S. sanguinis* showed remarkable bactericidal effect on 49% of indole-positive *Proteus* species (4/9 strains) and 60% of *Acinetobacter (A.) baumannii* (6/10 strains). We next investigated the property of bactericidal activity in filtrated supernatant by treating with proteinase K or autoclave. There was no change in the bactericidal activity of the filtrated supernatant after each treatment, excluding the involvement of protein and plasmid. Here, we identify the bactericidal activity in the filtrated supernatant of *S. sanguinis* against MDRP. This unexpected observation may contribute to the development of a novel therapeutic drug against *P. aeruginosa*. ——— Bactericidal effect; *Streptococcus sanguinis*; *Pseudomonas aeruginosa*; *Acinetobacter baumannii*; multidrug-resistant *Pseudomonas aeruginosa*.
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Streptococcus sanguinis (*S. sanguinis*) is one of the α-hemolytic streptococci that commonly reside in the human oral cavity. *S. sanguinis*, *S. mitis*, and *S. oralis* are categorized oral viridans. These bacteria, although less virulent (or non-pathogenic) in oral cavity, are the leading cause of infective endocarditis (Coykendall 1989; Ge et al. 2008; Kreth et al. 2008). *Pseudomonas aeruginosa* (*P. aeruginosa*) is a non-capsulate and non-sporing Gram-negative bacillus that most commonly affects the lower respiratory system associated with nosocomial infections. On the other hand, *P. aeruginosa* is medically important pathogenic bacteria, which can cause chronic pulmonary disease and other infections including pneumonia, sepsis, and urinary tract infection, especially in immunocompromised individuals. Cystic fibrosis is characterized by emergence and persistence of chronic infection, mostly *P. aeruginosa* that produces a surface polysaccharide known as alginate (Kukavica-Ibruli and Levesque 2008; Campodonico et al. 2008; Winstaney and Fothergill 2009). Antipseudomonal agents are available for controlling outbreak of *P. aeruginosa*. Low-dose macrolide therapy is commonly used to treat patients with chronic pulmonary infection, but there is some concerning the bacteria developing resistance to these drugs. Recently, treatment has become difficult due to nosocomial infections caused by multidrug-resistant *P. aeruginosa* (MDRP) (Basustaoglu et al. 1995; D'Agata 2004; Nouer et al. 2005; Rossoline and Mantengoli 2005; Bonomo and Szabo 2006; Paterson 2006; Hachem et al. 2007).

MDRP strains cause nosocomial infections with an increasing ratio in recent years, which have become important clinical problem; thus, the development of a novel therapeutic drug is expected. We report here the bactericidal activity in filtrated supernatant of *S. sanguinis* (NNSSH

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Correspondence: Masachika Senba, Department of Pathology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, 852-8523 Nagasaki, Japan.
e-mail: mikiyo@net.nagasaki-u.ac.jp

Table 1. *Pseudomonas aeruginosa* isolated from patients with diseases

No.	Age	Sex	Inpatient/Outpatient	Diagnosis	Underlying disease
1	25	m	inpatient	pneumonia	HIV
2	75	m	inpatient	pneumonia	sequelae of pulmonary tuberculosis
3	59	f	inpatient	chronic	DPB
4	80	m	outpatient	chronic	bronchiectasis, sequelae of pulmonary tuberculosis
5	77	m	inpatient	exacerbation	COPD
6	71	m	inpatient	exacerbation	COPD, sequelae of pulmonary tuberculosis
7	78	m	inpatient	chronic	pneumoconiosis
8	51	m	outpatient	pneumonia	bronchiectasis
9	78	f	inpatient	exacerbation	bronchiectasis
10	69	m	outpatient	exacerbation	COPD
11	83	f	inpatient	exacerbation	DPB, sequelae of pulmonary tuberculosis
12	74	f	outpatient	exacerbation	bronchial ectasis
13	77	f	outpatient	exacerbation	COPD, sequelae of pulmonary tuberculosis
14	74	m	outpatient	exacerbation	bronchial asthma, COPD
15	62	m	outpatient	exacerbation	pyothorax
16	81	f	outpatient	exacerbation	bronchiectasis
17	72	m	inpatient	chronic	COPD
18	74	m	inpatient	exacerbation	COPD
19	76	f	outpatient	exacerbation	bronchiectasis
20	77	m	outpatient	exacerbation	bronchiectasis

m, male; f, female; HIV, human immunodeficiency virus; DPB, diffuse panbronchiolitis; COPD, chronic obstructive pulmonary disease.

strain, isolated from our Department of Clinical Medicine) against multidrug-resistant, mucoid and non-mucoid types of *P. aeruginosa*.

Materials and Methods

Bacteria

A non-pathogenic strain of *S. sanguinis* isolated from a patient's sputum was used in this study. It is α-hemolytic, Gram-positive and resistant to optochin. The strain was identified using bacto-labo streptogram® according to the manufacturer's instructions (Wako, Osaka, Japan). In this experiment, we used a strain of *S. sanguinis* named 'NNSSH' that exhibited the strongest bactericidal function. NNSSH strain was isolated at our department from a patient. Moreover, variations in bactericidal effect were observed among *S. sanguinis* strains. Twenty strains of *P. aeruginosa* isolated from the sputa of 20 patients with pulmonary diseases, including 2 MDRP strains and 5 mucoid type strains, were used in this study (Table 1).

The bactericidal effect in filtrated supernatant of NNSSH strain of *S. sanguinis* against bacteria other than *P. aeruginosa* was also evaluated. Other bacteria strains used were 9 species: 10 strains of *Staphylococcus aureus* (*S. aureus*), 10 strains of *S. epidermides*, 10 strains of *Escherichia coli* (*E. coli*), 10 strains of *Klebsiella pneumoniae* (*K. pneumoniae*), 10 strains of *Enterobacter* species (8 strains of *Enterobacter cloacae*; 1 strain of *Enterobacter aerogenes*; 1 strain of *Enterobacter asburiae*), 10 stains of *Serratia* species (one strain of *Serratia liquefaciens*; 3 strains of *Serratia odorifera*; 6 strains of *Serratia morcescens*), 5 strains of *Citrobacter freundii*, 9 strains of indole-positive *Proteus* species (5 strains of *Providencia stuartii*; 3 strains of *Morganella morganii*; 1 strain of *Proteus vulgaris*), and 10 strains of *Acinetobacter baumannii* (Table 2).

Table 2. Bacteriocidal activity against various bacteria in *Streptococcus sanguinis* filtrated supernatant.

bacteria	bacteriocidal rate		
Staphylococcus aureus	0/10	strains	0%
Staphylococcus epidermidis	0/10	strains	0%
Escherichia coli	1/10	strains	10%
Klebsiella pneumoniae	0/10	strains	0%
Enterobacter spp.	0/10	strains	0%
Serratia spp.	1/10	strains	10%
Citrobacter freundii	0/5	strains	0%
indole-positive *Proteus* spp.	4/9	strains	44%
Acinetobacter baumanii	6/10	strains	60%
Pseudomonas aeruginosa	19/20	strains	95%

spp., species; *Enterobacter* spp. (8 strains of *Enterobacter cloacae*; 1 strain of *Enterobacter aerogenes*; 1 strain of *Enterobacter asburiae*); *Serratia* spp. (I strain of *Serratia liquefaciens*; 3 strains of *Serratia odorifera*; 6 strains of Serratia morcescens); *Proteus* spp. (5 strains of *Providencia stuartii*; 3 strains of *Morganella morganii*; 1 strain of *Proteus vulgaris*).

Filtrated supernatant of S. sanguinis

Several colonies of NNSSH strain of *S. sanguinis* from 7% rabbit blood agar were inoculated in 3 ml of Todd-Hewitt broth (Difco,

Detroit, MI, USA) and cultured overnight in static conditions. For negative control, Todd-Hewitt broth (3 ml) inoculated without bacteria was also cultured overnight in static conditions. In this study, aerobic condition was used for growth of *S. sanguinis*. They were subsequently centrifuged at 30,000 g for 30 min, and the supernatant was collected. This supernatant was filtrated with a commercially available filter (DISMIC®-25cs, 0.20 μm, Tokyo, Japan).

Treatment of P. aeruginosa

Each strain of *P. aeruginosa* was cultured overnight in 1 ml Muller-Hinton broth (Becton Dickinson Co., Sparks, MD, USA) at 35°C and then adjusted to 10^5 cfu/ml using physiologic saline. This solution (0.1 ml) was mixed with filtrated supernatant of NNSSH strain and incubated at 35°C under static conditions.

Determination of viable count of 20 strains of P. aeruginosa after treatment with filtrated supernatant of NNSSH strain

a) Viable count: Viable counts of 20 strains of *P. aeruginosa* were determined by the quantitative culture method on the first, second, fourth, fifth, and sixth day after treatment with the filtrated supernatant of NNSSH strain. Gram-stain was performed for confirmation of bactericidal effect.

b) Electron microscopic observation: Transmission electron microscopy (TEM) was used to observe any change that may have occurred on the structure of one smooth strain of *P. aeruginosa*. The sample was fixed for overnight at 4°C in a solution containing 0.1 M cacodylate buffer pH 7.3 of 2% gluteraldehyde, then fixed with 1.5% osmium tetroxide, and embedded in epoxy resin. Ultrathin sections were stained with lead citrate and uranyl acetate. All specimens for TEM were examined with a JEM-1230 (JEOL Ltd., Tokyo, Japan) electron microscope operated at 80 kV and photographed.

Evaluation of bactericidal effect

Sterile 0.9% NaCl was used as a diluent for preparing dilutions of *P. aeruginosa* from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. Bacterial growth in broth medium was observed usually turns the turbid medium. We evaluated the bactericidal effect in filtrated supernatant of NNSSH strain by using the minimum bactericidal concentration method (MBC). Thus, cultured broth medium was laid on BBL TSA II agar plate medium (Becton Dickinson Co., Sparks, MD, USA). The bactericidal effect was judged with or without bacterial colony on the agar plate medium. As a control, the bacteria were left untreated with filtrated supernatant of *S. sanguinis* (NNSSH).

Treatment with proteinase K or autoclave

The property of bactericidal component(s) was examined using proteinase K or autoclave treatment. Filtrated supernatant of NNSSH strain were treated with 0.1 ml of proteinase K (QIAGEN®, QIAamp DNA mini kit, Tokyo, Japan) at 50°C for 1 h to determine whether the bactericidal effect is medicated by a protein or not protein product. The enzyme was inactivated at 95°C for 10 min. Likewise, the filtrated supernatant was autoclaved at 121°C for 15 min to examine whether the bactericidal activity is heat-resistant or heat-labile.

Results

Effects of treatment with filtrated supernatant of NNSSH strain on the viable count of P. aeruginosa

All 20 strains of *P. aeruginosa* displayed viable count of 10^4 cfu/ml one day after treatment with filtrated supernatant of NNSSH strain. After 3 days, the viable count of 19 strains decreased to 10^3 to 10^2 cfu/ml, and the bacteria were observed at a small number. After 4 days, no viable bacteria were observed (Fig. 1). The bacteria growth was not

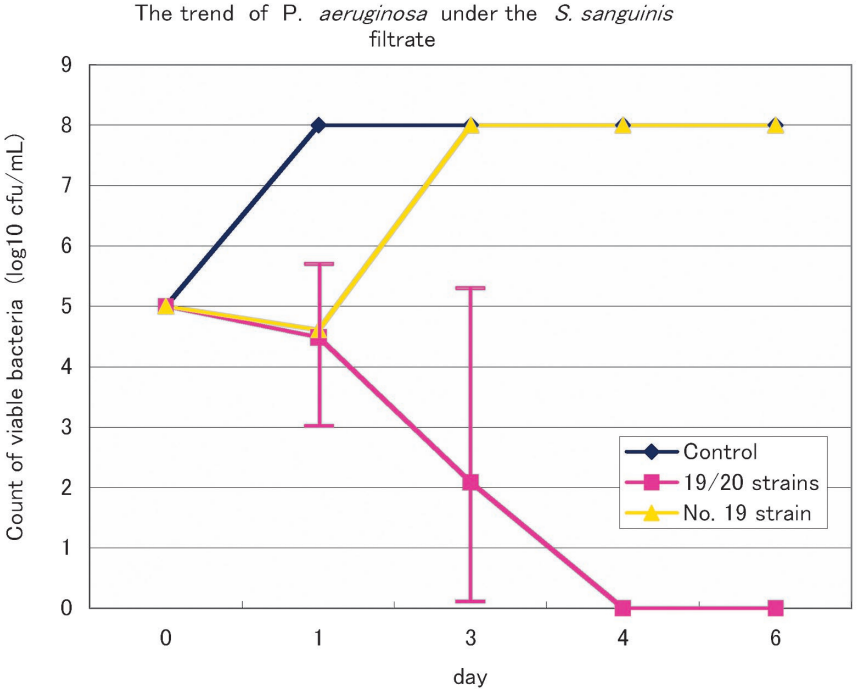

Fig. 1. Change in bacteria numbers of *Pseudomonas aeruginosa* treated with a filtrated supernatant of *Streptococcus sanguinis* strain (NNSSH). Bacteria were left untreated (control) or treated with a filtrated supernatant of *Streptococcus sanguinis* strain (NNSSH).

observed in 10^5 cfu/ml; on the other hand, broth turbid was observed in greater than 10^6 cfu/ml. In this study, 15 smooth strains and 5 mucoid strains were used. Of the 20 strains, one (5%), a mucoid strain of *P. aeruginosa*, was not affected by the treatment with the filtrated supernatant. Therefore, 19 strains of *P. aeruginosa* (15 smooth strains and 4 mucoid strains) were affected by bactericidal effect.

Morphological changes of cultured P. aeruginosa treated with filtrated supernatant of NNSSH strain

Gram-stain: After 3 days growth in the presence of filtrated supernatant of NNSSH strain, the characteristic morphological appearance was changed in *P. aeruginosa*. As shown in Fig. 2, the shape of bacteria was change from rod to oval.

Transmission electron micrography: Control *P. aeruginosa* culture density was deep and uniform (Fig. 3A). However, agglutination of bacterial somatic contents and fusional changes were seen in some areas of *P. aeruginosa* cultured in the presence of filtrated supernatant of NNSSH strain (Fig. 3B). After 3 days, these changes progressed remarkably (Fig. 3C).

Bactericidal effect on bacteria other than P. aeruginosa

Bactericidal effects were not observed with *S. aureus*, *S. epidermidis, E. coli*, *K. pneumoniae*, *Enterobacter* spp., *Serratia* spp., and *Citrobacter freundii*. The bactericidal effect was seen in 44% of indole-positive *Proteus* spp. and 60% of *A. baumannii* (Table 2).

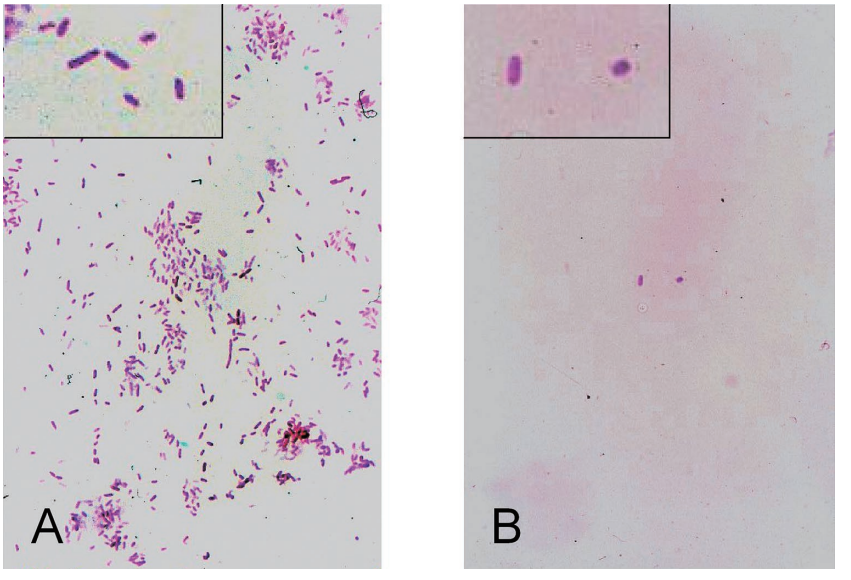

Fig. 2. Gram staining of *Pseudomonas aeruginosa*. A: Control: *Pseudomonas aeruginosa* without treatment of filtrated supernatant of *Streptococcus sanguinis*, B: *Pseudomonas aeruginosa* treated for 3 days with filtrated supernatant of *Streptococcus sanguinis*. The shape of bacteria was changed from rod (A) to oval (B). Also shown are bacteria under higher magnification.

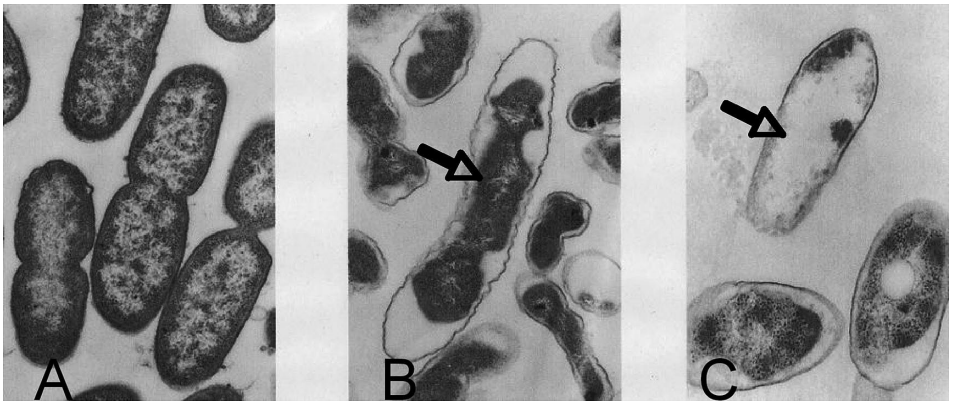

Fig. 3. Electron micrographs of *Pseudomonas aeruginosa*. A: Control: *Pseudomonas aeruginosa* without treatment of filtrated supernatant of *Streptococcus sanguinis*, B: *Pseudomonas aeruginosa* treated for one day with filtrated supernatant of *Streptococcus sanguinis*. Bacterial structure was denatured, associated with condensation (arrow). C: *Pseudomonas aeruginosa* treated for 3 days with filtrated supernatant of *Streptococcus sanguinis*. Denatured bacterial structure was gradually discomposed with time (arrow).

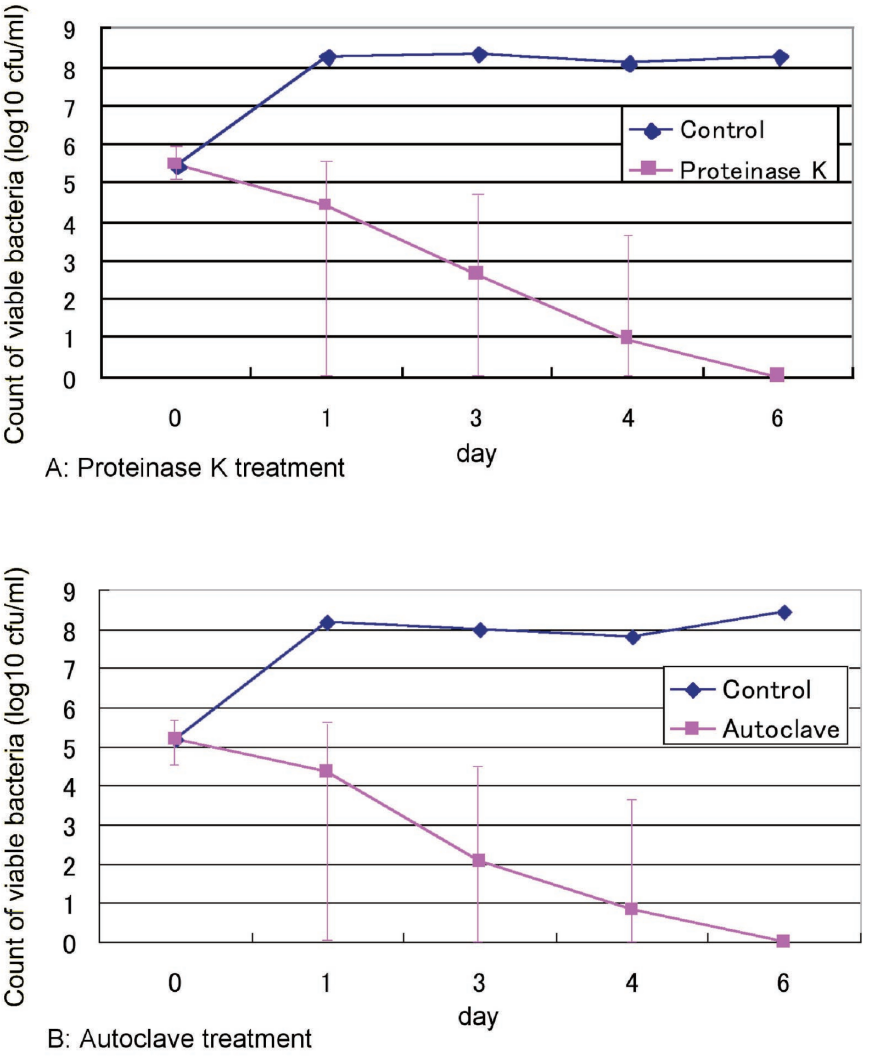

Fig. 4. Proteinase K and autoclave treatment of bactericidal component in filtrated supernatant of *S. sanguinis*. A: Proteinase K treatment. B: Autoclave treatment. After proteinase K or autoclave treatment, the bactericidal activity was preserved (see Fig. 1).

Proteinase K and autoclave treatment of filtrated supernatant of NNSSH strain

In order to further characterize the active bactericidal component of the filtrated supernatant of NNSSH strain, the filtrated supernatant was treated with proteinase K or autoclaved. After proteinase K treatment or autoclave treatment, the bactericidal effect was detectable (Fig. 4. A and B), which is similar to that of the untreated filtrated supernatant of NNSSH strain of *S. sanguinis* (see Fig. 1).

Discussion

The difficulty in effectively treating infections of highly resistant *P. aeruginosa*, especially MDRP, is a serious medical problem (Poole 2004). In intractable infection for the mucoid type *P. aeruginosa* under the most of patients with chronic respiratory disease cases, low-dose macrolide therapy may provide good results (Keicho and Kudoh 2002; Equi et al. 2002; Kudoh and Keicho 2003), but there is not enough effect of treatment. Colistin is the last choice for treatment of multidrug-resistant Gram-negative bacteria. Recently, colistin has been increasingly used in combination with one or more antibacterials for the treatment of MDRP patients. This combination therapy is used in order to improve the bactericidal activity for MDRP, despite the consequent increase in toxicity (Falagas and Kasiakou 2005; Li et al. 2006; Petrosillo et al. 2008). In a model of acute respiratory infection caused by *P. aeruginosa,* vaccination, a protein from a mucoid type *P. aeruginosa* was shown to effectively eliminate the bacteria from the lungs (Thomas et al. 2000).

On the other hand, concerning bacteriocin, Dajani and co-workers (1976) reported that they had clinically isolated 22 strains of *S. sanguinis*, and the filtrated solution had shown 29% of bactericidal effects for *P. aeruginosa*. The growth factor was inhibited by bacteriocin-like (viridin) that was a kind of the protein, and the substance loses the bactericidal activity by heating at 60˚C. They described the growth repression factor was viridin. In our study, the fil-

trated supernatant of *S. sanguinis* was observed bactericidal effect for *P. aeruginosa,* and our new-antipseudomonal substance was not destroyed by proteinase K and autoclave treatment. Therefore, the bactericidal component is completely different to Dajani's substance (Dajani et al. 1976). We are currently investigating the nature of the active bactericidal component present in filtrated supernatant of *S. sanguinis*. The activity was not altered after treatment with proteinase K and autoclave; thus, the bactericidal component is neither protein nor plasmid in nature. This bactericidal effect was observed on MDRP, both mucoid and non-mucoid types of bacteria, and the bactericidal rate indicated high as 95%, compared to 29% in the study of Dajani et al. (1976). We showed the electron microscopic changes of *P. aeruginosa* after treating with the antipseudomonal component. And we investigated that bactericidal component also have the antiactivity for *A. baumannii*.

The bactericidal component was also refined from the filtrated supernatant of cultured medium of oral viridans present in the normal flora of human oral cavity, which was observed unique as antipseudomonal component. The bactericidal effect attracted attention as pathogenic bacteria of the nosocomial infection. Recently, MDRP infection has become a serious problem clinically. Our urgent important task is to establish the novel alternative therapy for *P. aeruginosa*. Therefore, the bactericidal component will be developed as new treatment for *P. aeruginosa*. We believe that the active bactericidal component present in the filtrated supernatant of *S. sanguinis* offers a promising candidate for such therapies, and also we will investigate the way to make a new anti-pseudomonal drug in the near future.

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