Possibility of novel therapeutic strategy for multidrug resistant 
Pseudomonas aeruginosa using bactericidal activity in Streptococcus 
sanguinis secretion

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Multidrug-resistant Pseudomonas aeruginosa (MDRP) infection has become a serious clinical problem in the currently.
We reported the bactericidal activity in the filtrated supernatant of Streptococcus (S.) sanguinis against P. aeruginosa. The 
S. sanguinis, isolated from the sputum of a pulmonary-disease patient, was cultured for overnight, and filtered supernatant 
was tested for bactericidal effect using the minimum bactericidal concentration method. 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. P. aeruginosa were affected by bactericidal effect. The bactericidal activity was 
observed in the filtrated supernatant of S. sanguinis against MDRP. The high prevalence of MDRP requires discover 
pharmaceutical candidates. This new discovery phenomenon may contribute to the development of a novel therapeutic 
drug against P. aeruginosa.

Keywords bactericidal activity; Streptococcus sanguinis; Pseudomonas aeruginosa; multidrug-resistant Pseudomonas 
aeruginosa.

1. Introduction

Streptococcus coccus (S. sanguinis) is one of the a-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 [1-3]. Pseudomonas aeruginosa (P. aeruginosa) is a non-capuslate and non-sporing Gram-negative bacillus that most commonly affects the lower respiratory system associated with nosocomial infections. P. aeruginosa is a main cause of high morbidity and mortality 
among immunocompromised individuals. Thus, P. aeruginosa is medically important pathogenic bacteria, which can 
cause chronic pulmonary disease and other infections including urinary tract, cetrul nervous system, wound, eyes, skin, 
and musculoskeletal system infections, 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 [4-6]. Moreover, the resistance of P. aeruginosa to a variety of chemical compounds, including antibiotics, 
detergents, and hospital disinfectants facilitates, which is long-term persistence in the healthcare settings. The current 
care for treatment of P. aeruginosa infections is long-term use of antibiotics. The frequent use of antibiotics has led to 
widespread emergence of multidrug-resistant P. aeruginosa (MDRP) strains. 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 
have become difficult due to nosocomial infections caused by MDRP [7-13].

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 had reported the bactericidal 
activity in filtrated supernatant of S. sanguinis (NNSSH strain: isolated from our Department of Clinical Medicine, 
Institute of Tropical Medicine, Nagasaki University) against multidrug-resistant, mucoid and non-mucoid types of P. 
aeruginosa [14].

This mini-review focuses on technique to obtain of the bactericidal activity in filtrated supernatant of S. sanguinis 
against multidrug-resistant of P. aeruginosa and evaluation of bactericidal effect. Furthermore, the antibacterial effect 
of this production from the filtrated supernatant of S. sanguinis will help the development of a new therapeutic drug in 
the near future.

2. Technique for obtain of filtrated supernatant of S. sanguinis

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.

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, 3 ml of Todd-Hewitt broth 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).

3. Evaluation of bactericidal effect

3.1. Determination of viable count 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 P. aeruginosa strain (Fig. 1) and incubated at 35°C under static conditions.

![Fig. 1. Scanning electron micrograph of Pseudomonas aeruginosa (x10,000).](image)

Viable counts 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 Pseudomonas aeruginosa strain (Fig. 2 is result of NNSSH strain). Gram-stain was performed for confirmation of bactericidal effect (Fig. 3).

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 P. aeruginosa 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 (Fig. 4).
Fig. 2. 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 filtrated supernatant of *Streptococcus sanguinis* strain (NNSSH).

Fig. 3. Gram’s 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.
We evaluated the bactericidal effect in filtrated supernatant of *Pseudomonas aeruginosa* strain by using the minimum bactericidal concentration method (MBC). 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. Thus, cultured broth medium was laid on BBL TSA II agar plate medium. The bactericidal effect was judged with or without bacterial colony on the agar plate medium.

3.2. Morphological changes of cultured *P. aeruginosa*.

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 0.1M 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 (Fig. 5).
4. Discussion

*P. aeruginosa* is an important pathogen causing a wide range of acute and chronic infections. *P. aeruginosa* has a high mortality compared with other pathogens. In addition, *P. aeruginosa* is the most prevalent chronic infection contributing to the pathogenesis of cystic fibrosis. The difficulty in effectively treating infections of highly resistant *P. aeruginosa*, especially MDRP, is a serious medical problem [15]. 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 [16-18], 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 [19-21]. 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 [22].

The cystic fibrosis is the most common genetic cause of early death, and inflammatory lung disease continues to be the major cause of morbidity and mortality. As nuclear factor-κB (NF-κB) dependent cytokines and chemokines are major mediators of lung inflammation and destruction in cystic fibrosis, antagonists of the production and/or actions of these cytokines would be expected to be beneficial [23]. The NF-κB family of transcription regulatory proteins has been implicated in the control of a variety of inflammatory processes. NF-κB influences production of a variety of proinflammatory cytokines that appear to be involved in the pathogenesis of cystic fibrosis lung disease. NF-κB is activated when its inhibitor, IκB, is phosphotylated by IκB Kinase (IKK), then ubiquitinated and degraded. The free NF-κB translocates to the nucleus, binds to promters of NF-κB dependent genes, and facilitates their transcription. NF-κB may be an important target for new anti-inflammatory approaches for treating cystic fibrosis lung disease [24]. Therefore, there is evidence for a hyperinflammatory condition in cystic fibrosis that may be multifactorial and related to structural changes with increased mucus viscosity, increased activation of inflammatory pathway, such as NF-κB, and/or a decrease in anti-inflammatory mediators [25, 26].

In our previous studies, All 20 strains of *P. aeruginosa* displayed viable count of $10^7$ cfu/ml one day after treatment with filtrated supernatant of NNSSH strain. After 3 days, the viable count of 19 strains decreased to $10^5$ to $10^6$ 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 observed in $10^7$ cfu/ml; on the other hand, broth turbid was observed in greater than $10^8$ 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 [14]. By 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 Figure 2, the structure of the bacteria was change to oval in shape, with both ends unfilled. By transmission electron micrography, Control *P. aeruginosa* culture density was shown deep and uniform (Fig. 5A). 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. After 3 days, these changes progressed remarkably (Fig. 5B).

Concerning bacteriocin, Dajani and co-workers (1976) [27] 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 filtrated 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 [14]. Therefore, the bactericidal component is completely different to Dajani's substance (Dajani et al. 1976) [27]. We are currently investigating the nature of the active bactericidal component present in filtrated supernatant of *S. sanguinis* strain. 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 in 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. *P. aeruginosa* outbreaks among immunocompromised patients represent dangerous events. In our recent study, the material of the bactericidal effect was not identified, however, it was suggested that the material included the sugar chain (unpublished data). 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* strain 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.

5. Appendix: Detection of *P. aeruginosa* in paraffin embedded human tissue

The author applied Warthin-Starry procedure for identification *P. aeruginosa*. The bacteria were cultured for identification of organisms, and were confirmed as *P. aeruginosa*. The marials were fixed in 10% formalin, embedded in paraffin, and cut at routinely.

**Procedure of the staining as following:** The steps involved in modified Warthin-Starry method are the following: (1) Deparaffinized and hydrate to distilled water. (2) Treat with 0.1% citric acid buffer (pH 4.0) using 2 cycle changes of 3 minutes each. 0.1% citric acid buffer solution: In 100 ml of distilled water, dissolve 0.1 g citric acid. (3) Impregnate with silver nitrate solution and keep at 60°C in an incubator for 30 to 60 minutes. Silver nitrate solution for impregnate: In 100 ml of 0.1% citric acid buffer as above (step 2), dissolve 2 g of silver nitrate. (4) Treat with developer solution for 2 to 7 minutes. This solution must be prepared fresh just before use. Allow sections to develop until they are light brown to yellow. Check known control under the microscope. Develop solution: Mix 38 ml of 5% gelatin in 0.1% citric acid buffer as above (step 2), both previously heated at 60°C, and add 0.15% hydroquinone in 0.1% citric acid buffer as above (step 2). Mix and used immediately. (5) Wash quickly and thoroughly with warm water at 55 to 60°C and then with distilled water. (6) Dehydrate, clear and mount.

**Results of the staining as following:** *P. aeruginosa* were stained intense black to dark brown (Fig. 6). Connective tissue was stained dark brown to yellow. Background was stained yellow to light brown. Nuclei, melanin, certain hematogenous pigments was stained intense black for the silver particles. Overdevelopment gives dark background. On the other hand, underdevelopment gives pale background and pale bacteria. In a case, optional development gives good condition.

**Comments of the staining as following:** The modified Warthin-Starry silver impregnation method was a reliable alternative for Gram’s stain procedure for identification of *P. aeruginosa* in paraffin embedded tissue sections. The Warthin-Starry method is most commonly used for the staining of spirochetes, such as *Triponema pallidum, Borrelia*, and *Leptospira*. This method has also proved useful for the demonstration of the causative agent of Legionella pneumophila as well as spirochetes [28].

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Numerous *Pseudomonas aeruginosa* bacteria are demonstrated in an autopsy case of pneumonia caused by *P. aeruginosa* from the human lung tissue. This method has proven superior to others in demonstrating a reliable staining for *P. aeruginosa*. Lung injury associated with *P. aeruginosa* infection results from both the direct destructive effects of the organism on the lung parenchyma and exuberant host immune responses. Modified Warthin-Starry method.

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**References**