

Adherence of *Pseudomonas aeruginosa* to a Rabbit Cornea Cell Line (SIRC) Cells*

Takashi IIDA¹⁾, Masafumi KATOH²⁾ and Yoshiyasu MATSUO²⁾

1) Department of Ophthalmology, Hiroshima University School of Medicine, Hiroshima 734, Japan

2) Department of Bacteriology, Hiroshima University School of Medicine, Hiroshima 734, Japan

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ABSTRACT

Adherence of *Pseudomonas aeruginosa* to a Rabbit Cornea Cell Line (SIRC) cells *in vitro* was examined. Maximal adherence occurred at pH 5.0-7.0 and when the bacterial suspension at 1.4×10^9 /ml was exposed to 2×10^4 /ml SIRC cells at mid-logarithmic growth phase. Adherence was not affected by temperature. In the passage of time, a temporary rise of adherence occurred within 5 min of incubation and stationary phase was maintained from 30 min through 90 min of incubation. Killing of *P. aeruginosa* with heating at 65°C for 30 min or 60 min resulted in a marked decrease in adherence. Similar results were obtained by pretreatment of bacteria with 3% formaldehyde. Different strains of *P. aeruginosa* varied in their abilities to adhere to SIRC cells. Strains which produce protease adhered more avidly to SIRC cells than those which produce no protease, while the presence of pili was not correlated with bacterial adherence.

INTRODUCTION

In recent years it has become widely recognized and accepted by many investigators that the adherence of bacteria to mucosal epithelial surface was an initial stage in bacterial infections. Many researches have been done on the adherence of pathogenic bacteria to the epithelial cells on gastrointestinal^{1,15)} and genitourinary tracts^{4,5,25,26,30,33,39)}, oral cavity^{8,23,38)}, and respiratory tract^{21,31,36)}. Among them, studies on the adherence of *Escherichia coli*^{4,5,25,26)}, *Neisseria gonorrhoeae*^{10,32,33)} or streptococci^{1,2,8,17,39)} have revealed the mechanism of adherence in detail. Bacteria-host cell interactions are based on some kinds of attractive force between the surfaces of both bacteria and epithelial cells, which contain a non-specific force such as surface hydrophobicity, net surface charge and DLVO theory¹⁹⁾ (long-range attractive force which interacts between negatively charged lyophobic colloid particles), and specific ones such as ligand (adhesin)-receptor interactions. Ligands are generally known to be pili in gram-

negative bacteria^{5,6,11,14,22)} and lipoteichoic acids in gram-positive bacteria^{2,17)}. On the other hand, receptors on epithelial cells are mostly identified with sugar moieties^{18,25,26)}.

The adherence of *P. aeruginosa* to tracheal^{21,36)} or buccal epithelial cells^{34,35)} has been studied by some workers. Woods et al. previously demonstrated that pili mediated the adherence of *P. aeruginosa* to buccal epithelial cells, and pointed out in their recent papers^{35,36)} that cell surface fibronectin might prevent the organism from the adherence. Since fibronectin is easily destroyed by protease³⁷⁾, protein-degrading enzymes produced by *P. aeruginosa* may behave as a potent adherent factor of the bacilli.

The purposes of the present study are to characterize the adherence of *P. aeruginosa* to a Rabbit Cornea Cell Line (SIRC) cells and to gain information about the correlation between the attachment and pili or protease on the bacterial binding site.

MATERIALS AND METHODS

Bacteria. Twenty-eight strains of *P. aerugi-*

*) 飯田貴士, 加藤雅史, 松尾吉恭: 緑膿菌の家兎角膜培養細胞への付着性について

nosa were subjected to the study. The strain 2077 (NCTC 10490) was a stock culture in our laboratory. Strains IFO 3455, NC 5, and PA 103 were kindly supplied by Dr. T. Nishio, Hiroshima Prefectural Institute of Public Health. Strains 184, 186, 198, 226, 279, 304, 397, 455, 474, 561, 595, 638, 670, 690, 694, 766, 831, 835, 871, 887, 898, 942, 969, and 989 were clinically isolated from Hiroshima Prefectural Hiroshima Hospital from November, 1980 to November, 1981⁹. All the organisms were maintained on slopes of trypticase soy agar. Before testing, the bacteria were transferred to trypticase soy broth and incubated for 24 hr at 37°C. The organisms were harvested by centrifugation, washed three times with Dulbecco's phosphate buffered saline (PBS) and resuspended in the same buffer. The concentration of the bacterial suspension was adjusted to an optical density of 0.5 at 570 nm (approximately 1.4×10^9 /ml) on Coleman Junior spectrophotometer.

Epithelial cell culture. The SIRC cell was established from the cornea of a normal rabbit by M. Volkert at Staatens Serum-Institute, Copenhagen, Denmark in 1957. The cells were routinely cultured in Dulbecco's modified essential medium (D-MEM; Nissui Seiyaku Co., Ltd., Japan) supplemented with 10% calf serum (Grand Island Biological Co., U.S.A.), per ml 100 U of penicillin and 100 µg of streptomycin.

When the cells developed into a confluent monolayer, they were detached by 0.2% trypsin and 0.02% EDTA in PBS, washed once and suspended in the culture medium without adding antibiotics at a density of 2×10^4 cells per ml. A 1 ml-portion of the cell suspension was allowed to settle on coverslips in multi-well tissue culture plates (Falcon 3008; Becton, Dickinson & Co., U.S.A.) and incubated overnight in a 5% CO₂ atmosphere at 37°C.

Adherence assay. The adherence of *P. aeruginosa* to SIRC cells *in vitro* was examined by replacing the overnight-cultured medium with the bacterial suspension. The cells exposed to the bacteria were incubated statically at 37°C for 60 min. After the incubation, the coverslips were washed three times with PBS on a rotary shaker (100 rpm) to remove non-adherent bacteria. The cells were fixed with absolute methanol for 5 min and stained with 0.02% crystal violet in water. The mean number of bacteria

attached per cell was determined by direct microscopic enumeration of 50 SIRC cells. All the experiments were performed in duplicate.

Electron microscopy. 1) The bacteria were suspended in a drop of distilled water mounted on a supporting membrane. The excess fluid was removed with a filter paper and dried at 37°C for 10 min. Then they were completed by chromium-shadowing. The specimens were examined to investigate the presence of pili on the bacteria with a transmission electron microscope (JEM-200CX, JEOLCO, Tokyo, 80KV). 2) The SIRC cells on glass slides (5×5×1 mm), to which the bacteria had already attached themselves as described above, were fixed with 2% glutaraldehyde, dehydrated with acetone and dried by the critical point drying technique. Then, the samples were coated with Au-Pd and examined with a scanning electron microscope (JSM-F7, JEOLCO, Tokyo, 7KV). 3) A mixture of 1 ml of the bacterial suspension (1.4×10^9 /ml) and an equal volume of SIRC cell suspension (2×10^4 /ml) were incubated at 37°C for 60 min. After incubation, the mixture was washed twice in PBS by centrifugation. Then samples were fixed by double treatment, 2% glutaraldehyde and 1% osmium tetroxide, at 4°C overnight each. After embedding by 1.5% agar, the small block was embedded into Epok 812. The ultrathin sections were obtained by cutting with ultratome, and doubly stained by uranyl acetate and lead citrate. The completed specimens were examined with a transmission electron microscope (JEM-200CX, 80KV).

Serotype and enzyme production of *P. aeruginosa*. Serotype and enzyme production of *P. aeruginosa* used in this study were referred from our previous report⁹.

RESULTS

General conditions for adherence. When the strain 2077 of *P. aeruginosa* was incubated with SIRC cells at 37°C, the maximum adherence was observed within 5 min of incubation as shown in Fig. 1. The number of attached bacteria gradually decreased until 30 min, and was maintained at a stationary level throughout 90 min of incubation.

The adherence of the bacteria was examined at four different bacillary growth phases. Table 1 demonstrates that maximal adherence was predominant at the mid-logarithmic and early

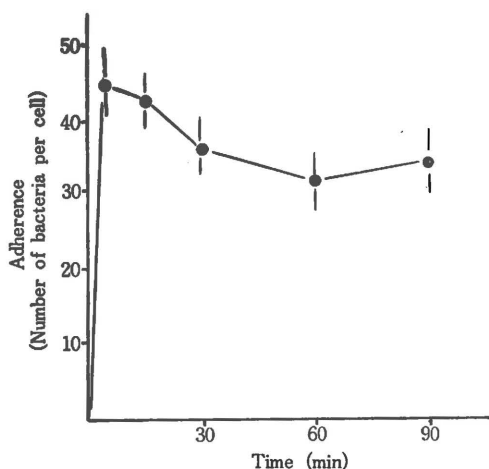


Fig. 1 Effect of incubation time on adherence of *P. aeruginosa* strain 2077 to SIRC cells. Adherence assay was carried out at 37°C. Bars indicate mean \pm standard error.

Table 1. Adherence of *P. aeruginosa* strain 2077 at various phases of bacterial growth

Age of culture (hr)	Growth phase	Mean no. of bacteria per cell \pm S. E. ^{a)}
12	Early-log	24.7 \pm 2.0
24	Mid-log	34.9 \pm 4.0
48	Early stationary	32.0 \pm 2.5
72	Late stationary	26.2 \pm 1.7

a) standard error.

stationary phases. However, no significant difference was observed among these growth phases.

The relationship between bacterial concentration and adherence was examined by adding various concentrations of the strain 2077 to a constant number of SIRC cells. Bacterial concentrations tested ranged from 1.4×10^6 /ml to 1.4×10^{10} /ml. Fig. 2 shows that the maximum adherence occurred at the concentration of 1.4×10^9 /ml.

Based on the above findings, conditions for adherence assay in subsequent experiments were decided as follows: per ml 1.4×10^9 bacteria at mid-logarithmic phase and incubation with SIRC cells at 37°C for 60 min.

Fig. 3 shows the adherence of *P. aeruginosa* to SIRC cells observed by scanning or transmission electron microscopy. Fig. 3-A is a scanning electron micrograph, in which the penetration and attachment of the bacteria to the

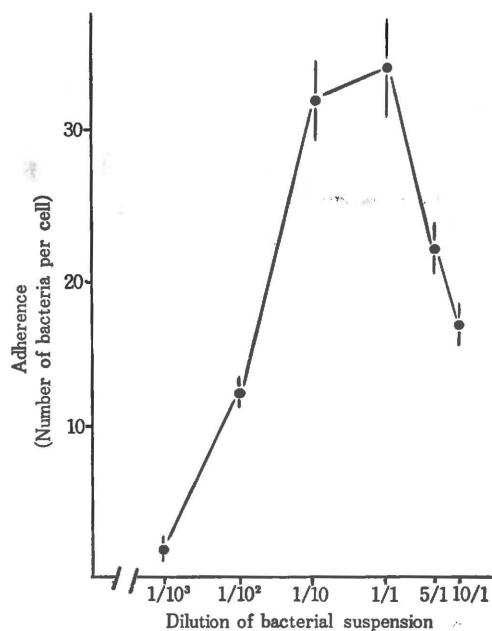


Fig. 2 Effect of bacterial concentration of *P. aeruginosa* strain 2077 on adherence to SIRC cells. Dilution at 1/1 was equivalent to the concentration 1.4×10^9 /ml. Bars indicate mean \pm standard error.

surface of the host cell can be seen (Arrow-a, -b). For example, these phenomena are observed as the ends of cells (a) are invaginating into dents and as the ends of cells (b) are attaching themselves to the surface of the host cell. Fig. 3-B is a transmission electron micrograph, indicating a close attachment between a bacteria cell body and host cell surface. No remarkable change can be seen in the cytoplasm of both cells and at the adhesion site (arrow).

Effect of temperature and pH on adherence. To determine the effect of temperature on adherence, the strain 2077 and SIRC cells were incubated at 4, 22, or 37°C for 60 min. Adherence at 4, 22, or 37°C was almost constant (Table 2). The strain 2077 and SIRC cells

Table 2. Effect of temperature on adherence of *P. aeruginosa* strain 2077 to SIRC cells

Temperature	Mean no. of bacteria per cell \pm S. E. ^{a)}
4°C	34.8 \pm 2.8
22°C	28.7 \pm 2.5
37°C	32.6 \pm 2.0

a) standard error.

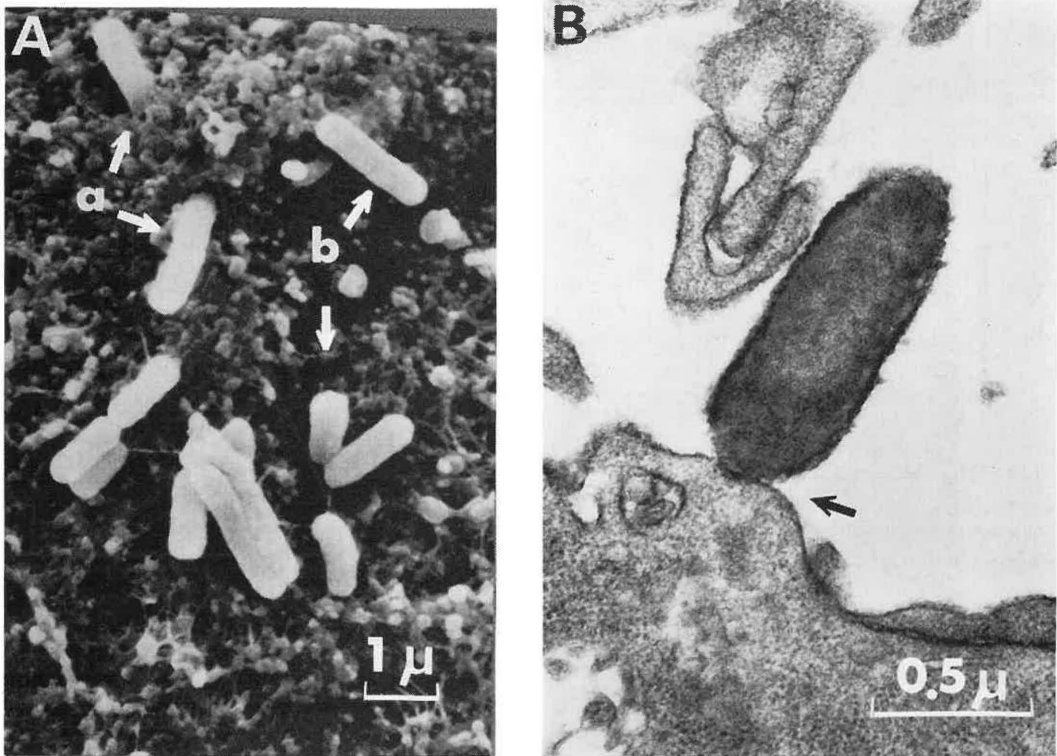


Fig. 3 Adherence of *P. aeruginosa* strain 2077 on the surface of SIRC cells: (A) scanning electron micrograph and (B) transmission electron micrograph.

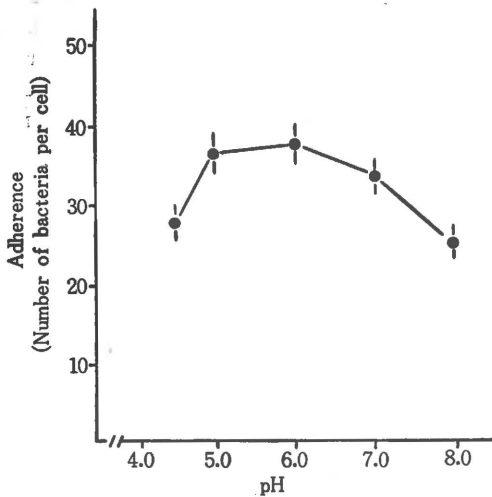


Fig. 4 Effect of pH of incubation medium on the adherence of *P. aeruginosa* strain 2077 to SIRC cells. Adherence assay was carried out in 20 mM phosphate buffered-saline with Ca^{++} , Mg^{++} at pHs of 4.5, 5.0, 6.0, 7.0 and 8.0 for 60 min at 37°C. Bars indicate mean \pm standard error.

were incubated at 37°C for 60 min in PBS at

pHs ranged from 4.5 to 8.0. As shown in Fig. 4, adherence appeared to be better at pHs from 5.0 to 7.0.

Effect of bacterial killing. Table 3 summarizes the adherence of the strain 2077 killed by heating or formaldehyde treatment. These procedures significantly decreased the ability of the organisms to adhere to SIRC cells.

Table 3. Effect of bacterial killing on adherence of *P. aeruginosa* strain 2077 to SIRC cells

Method of killing	Treatment	Mean no. of bacteria per cell \pm S. E. ^{a)}
Heat	None	34.6 \pm 3.1
	65°C 30 min	10.5 \pm 0.6
	65°C 60 min	4.7 \pm 0.5
3% formaldehyde	None	37.9 \pm 3.1
	37°C 30 min	2.6 \pm 0.4

a) standard error.

Effect of washing of bacteria. The strain 2077, which had been confirmed to have pili, was washed several times and adherence assay

was performed. As shown in Table 4, washing of the bacteria before incubation with SIRC cells made no significant difference on bacterial adherence.

Adherence of different strains of P. aeruginosa to SIRC cells. Twenty-eight strains of *P.*

aeruginosa were studied for their adherence ability to SIRC cells (Table 5 and Fig. 5). Protease-producing strains adhered more to SIRC cells than non-producing ones: the difference was significant ($p < 0.001$)

No correlation was observed on bacterial adherence with the presence of pili of the organism.

Table 4. Effect of washing of bacteria on adherence of *P. aeruginosa* strain 2077 to SIRC cells

Treatment	Mean no. of bacteria per cell \pm S. E. ^{a)}
1 washing	28.9 \pm 2.2
3 washings	36.5 \pm 2.0
6 washings	30.8 \pm 1.8

a.) standard error.

DISCUSSION

In this report, we demonstrated the adherence of *P. aeruginosa* to SIRC cells. Optimal conditions were as follows: incubation time, 60 min; bacterial concentration, 1.4×10^9 /ml; age of bacteria, mid-logarithmic phase; pH of incubation medium, 5.0-7.0. Generally, temporal

Table 5. Adherence of different strains of *P. aeruginosa* to SIRC cells

Strain	Serotype	Pili	Enzyme production		Adherence ^{a)}
			protease	elastase	
184	E	+	+	+	19.3 \pm 1.3
186	G	+	-	-	18.7 \pm 1.1
198	A	+	+	+	38.2 \pm 2.5
226	G	+	+	+	48.7 \pm 3.2
279	B	+	+	+	30.2 \pm 2.7
304	B	+	+	+	34.2 \pm 2.4
397	E	+	-	-	9.4 \pm 1.0
455	E	+	+	+	31.8 \pm 1.6
474	NT ^{b)}	+	+	-	58.0 \pm 2.7
561	NT	+	-	-	13.1 \pm 1.0
595	B	+	+	-	52.8 \pm 3.8
638	B	+	+	-	36.5 \pm 2.7
670	G	+	+	+	56.3 \pm 3.1
690	NT	+	+	-	22.3 \pm 1.6
694	E	+	+	-	38.5 \pm 2.1
766	E	+	-	-	11.9 \pm 1.1
831	G	+	-	-	18.0 \pm 1.2
835	G	+	-	-	8.9 \pm 0.7
871	B	+	+	-	52.1 \pm 3.3
887	E	+	+	+	38.2 \pm 3.5
898	E	+	+	-	29.2 \pm 1.9
942	H	+	-	-	20.7 \pm 1.4
969	NT	+	+	-	40.4 \pm 3.4
989	B	+	+	-	35.3 \pm 2.6
2077	A	+	+	+	39.6 \pm 2.4
IFO 3455	G	+	+	+	39.7 \pm 2.3
NC 5	E	+	-	-	8.1 \pm 1.0
PA 103	E	+	-	-	6.3 \pm 0.7

a) Mean no. of bacteria per cell \pm standard error.

b) Non-typable

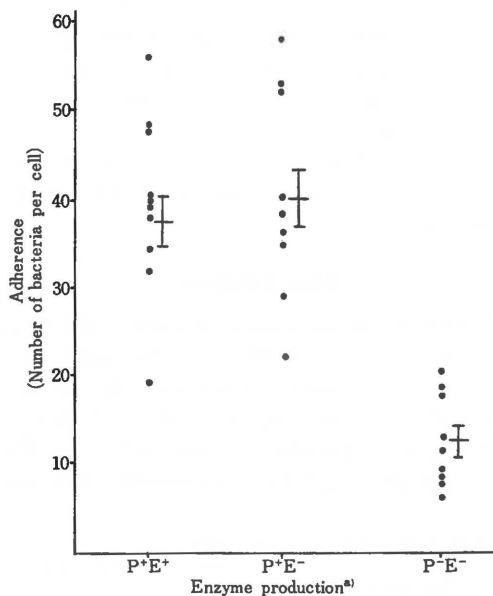


Fig. 5 Relationship between enzyme production and adherence of *P. aeruginosa* to SIRC cells
 a) P⁺E⁺: strains producing protease and elastase; P⁺E⁻: strains producing protease only; P⁻E⁻: strains producing no enzymes. Bars indicate average no. of adherence in various strains according to enzyme productions \pm standard error. Average number of bacteria per cell was 37.6 ± 3.0 (n=10) in P⁺E⁺, 40.6 ± 3.7 (n=9) in P⁺E⁻, and 12.8 ± 1.6 (n=9) in P⁻E⁻.

process of adherence is divided into two main types. The first, observed with *E. coli* adherence to human intestinal cells⁹⁾, is that adherence gradually increases according to time and reaches stationary level. This phenomenon shows that binding sites on epithelial cells are limited and demonstrates the presence of receptors. The second, observed with *Eikenella corrodens* adherence to human buccal cells⁸⁸⁾, is that adherence rapidly increases to maximal level and decreases gradually to stationary level. Our data corresponded to the latter. Shaeffers et al.²⁵⁾ explained that adherence might involve temporary binding in its early phase and stable form of attachment. Bacterial concentration needed in our study was greater than those reported by previous investigators^{1, 25, 89)}. This may be due to the fact that our adherence assay was done in static condition without agitation or mild centrifugation. Adherence of *P. aeruginosa* to SIRC cells seemed to be dependent on pH and be enhanced in weak

acidic environment, similar to adherence of *E. coli*^{4, 25)} and group B streptococci⁸⁹⁾.

The adherence of bacteria to epithelial cells has been studied by many investigators. Several works using phenotypic variants of bacteria clarified correlation between bacterial adherence *in vitro* and infectivity *in vivo*^{12, 24, 27-29, 32)}. These studies also revealed the mechanism of adherence. Much evidence has been presented to suggest that adhesive sites exist on the surface of bacteria and complementary sites on the surface of host cells. Pili, which are possessed by many species of gram-negative bacteria, are well recognized to mediate not only hemagglutination but also bacterial adherence to epithelial cells²⁰⁾. Eden and Hansson⁵⁾ noted a relationship between the presence of pili on *E. coli* and ability of the organism to adhere to human uroepithelial cells. Similar observations were made with *Proteus mirabilis*^{28, 29)} and *Neisseria gonorrhoeae*^{10, 32, 33)}.

Studies on the adherence of *P. aeruginosa* to respiratory tract^{21, 36)} and buccal epithelial cells^{34, 35)} have previously been reported. The presence of pili on *P. aeruginosa* was demonstrated by a number of investigators^{21, 34, 36)}.

Woods et al. definitely mentioned that pili mediated the adherence of *P. aeruginosa* to human buccal epithelial cells³⁴⁾. However, the results in our study showed that the adherence of *P. aeruginosa* to SIRC cells was not directly correlated with pili, since the bacilli washed vigorously several times for the purpose of depilation did not lose their adherence ability. *P. aeruginosa* killed with heating or formaldehyde treatment lost the ability to adhere to SIRC cells. These treatments of the bacilli cause not only the loss of pili but also diminishing protease activity or production. Woods et al.³⁵⁾ recently reported that pretreatment of human buccal cells with protease derived from *P. aeruginosa* or trypsin resulted in increased adherence of the organism and that immunofluorescent technique demonstrated little or no fibronectin in trypsinized epithelial cells or cells attached with a large number of the bacilli. They explained that cell surface fibronectin had an important role in adherence of *P. aeruginosa* and that the presence of fibronectin prevented the organisms from adhering to buccal cells. Cell surface fibronectin is a glycoprotein and easily digested by proteases such as trypsin.

Our data suggest that protease production of *P. aeruginosa* is closely correlated with the adherence. However, protease produced by *P. aeruginosa* has optimal pH 8.0 to 9.0 with casein¹⁶). The present study showed that optimal pH of the adherence was more acidic than that of the optimal enzyme reaction, and further that the adherence occurred adequately even at 4°C. These findings are in contrast to an assumption that protease might play an important role in bacterial adherence of *P. aeruginosa*, and suggest the existence of other factors which determine the adherence.

Although *P. aeruginosa* is a weak pathogen, it causes sometimes serious infections such as infection after burn, corneal infection, bleeding pneumonia and postsurgical infection. Virulence factors of the organism have been considered to be proteolytic enzymes and exotoxin A. Especially, corneal ulcer caused by *P. aeruginosa* is closely related with the bacterial enzymes. Kawaharajo¹⁸) demonstrated that *P. aeruginosa* strains producing protease and elastase had stronger virulence in experimental mouse corneal ulcer than those producing no enzymes. Further investigation is needed on enzyme production of *P. aeruginosa* regarding the bacterial virulence and adherence.

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