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Identification of Pathogenicity of *Pseudomonas Aeruginosa* by Caseinase Test

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ABSTRACT

According to their responses on 2% skim milk heart-infusion agar plate, Pseudomonas aeruginosa were classified into three groups. The A group made a clear zone of complete hydrolysis around the colony and a cloudy precipitate zone immediately around the clear zone; the B group had only a cloud precipitate zone around the colony and the C group did not have caseinolytic activity. The distribution of 48 clinically isolated strains in A, B and C groups was 72.9%, 20.8% and 6.3%, respectively.

Comparing the mortality of mice challenged with bacterial cells and the exotoxin products, we found that A and B groups had similar virulence and the C group did not have pathogenicity. By NTG-treated caseinase-deficient mutants, we also proved that the infectivity and toxigenicity were related to their producing ability of caseinase, but had no relationship with that of lecithinase, elastase, DNase, and with their pyocyanin production. The injection route also affected the virulence of P. aeruginosa to mice.

Key words: Pseudomonas aeruginosa, virulence, caseinase, toxigenicity.

Pseudomonas aeruginosa is widely distributed in our environment. They are the normal flora in mammalian intestine and also found on the human skin. It is an opportunistic pathogen and a leading cause of infection in hospitalized patients, when host defenses have been decreased by serious illness and aggressive chemotherapy[1-4]. *P. aeruginosa* could cause cystic fibrosis and serious lung infection[5-6]. The pathogenic factors come from many extracellular products, such as exotoxin; protease; hemolysins; pyocyanin and endotoxin etc.[1,6-13].

The presence of lecithinase and elastase was often used to identify the pathogenic strains of *P. aeruginosa*[14-15]. Serological typing, phage typing and pyocin

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typing have been used for epidemiological investigations and discussion of the differences between *P. aeruginosa* strains. We have reported that the presence of caseinase in *Staphylococcus aureus* and *S. epidermidis* was closely associated with its pathogenicity[17]. So we planed to use the clinically isolated *P. aeruginosa* to study their hydrolytic activity on the skim milk heart-infusion agar. Then we would discuss the relationship between their caseinase producing ability and pathogenesis to mice. By using the NTG-treated caseinase-deficient mutants, we found that the caseinase producing ability was closely related to their invasiveness and toxigenicity to mice. So we might use the caseinase producing ability to observe the pathogenesis of *P. aeruginosa.* It was a simple and convenient method of identification and could replace animals experimental.

MATERIALS AND METHODS

Tested strains

The *Pseudomonas aeruginosa* were obtained from *Taipei Municipal Chung-Hsing Hospital's* clinical specimen (1985), isolated by nalidixic acid cetrimide agar (NAC; Eiken) and identified by the following biochemical characters according to *Koneman et al.*[18], these biochemical test include the producing ability of indophenol oxidase, lysine decarboxylase, ornithine decarboxylase and arginine hydrolase; the oxidations of maltose, mannitol and gluconate; nitrate reduction; pyocyanin production (Pseudomonas P & F media were used; Difco); the ability of growing on 42°C in nutrient broth. The identified bacteria were kept in semisolid nutrient medium (Difco).

The measurement of lecithinase, elastase, DNase and caseinase

The lecithinase testing medium was prepared with NaCl 5.0 g, K_2HPO_4 5.0 g, Bacto-agar 15.0 g, egg yolk 100 ml, distilled water 900 ml. After incubation at 37°C for 48 hrs, strains of positive lecithinase formed a zone of insoluble precipitate in the medium surrounding the bacterial colonies[14-15, 19-20].

The measurement of elastase was carried out according to *Hedeberg's* elastase agar tube method[16]. The medium composition was elastine-Congo red (Sigma) 1%, NaCl (Wako Chemicals) 0.85% and agar (Difco) 1.5%. The medium was dissolved by heating before dispensing in Durham's tube (15 mm high). After autoclaving, the preparation was mixed well and cooled immediately to prevent the precipitation of elastine. 0.1 ml of 18-hrs heart-infusion (Difco) culture broth was inoculated on the tube surface. After incubation at 35°C for 72-hrs, a positive test of elastase was development of a clear zone which was clearly different from the original red particles.

DNase testing medium was prepared with heart extract (Nissan Chemicals) 1%, polypeptone (Kyokuto Chemicals) 1%, DNA (N.B. Co.) 0.2%, NaCl (Wako Chemicals) 0.5%, $CaCl_2$ (Wako Chemicals) 0.04% and agar (Difco) 1.5%. All the ingredients were dissolved in distilled water, autoclaved at 121.5°C for 15 min and poured into

petri dishe to make a DNase testing agar plate. P. aeruginosa was cultured on plates by band-inoculation. After 24-hrs incubation at 37°C, the cultures were flooded with 1.5 N HCl. A clear halo around the colony streak greater than 2 mm from the edge of colony indicated a positive reaction[21].

The skim milk heart-infusion agar was used for the test of caseinase[17]. Skim milk (Difco) and heart-infusion agar (Difco) were autoclaved separately, mixed well and then poured into petri dishes to make 2% skim milk heart-infusion agar. After 24-hrs incubation, the formation of a clear zone or cloudy precipitate halo around the bacterial colonies indicated positive for caseinase production.

The induction of caseinase mutants[22]

The parental strain was cultured in heart-infusion broth (Difco) for 18 hrs, and then washed with sterile normal saline for 3 times. The bacteria suspension (The concentration was about MacFarland nephelometer Standards No. 2) was treated with N-methyl-N'-nitro-N-nitrosoguanidine (Sigma; NTG) 50 μg/ml of 30 minutes. The NTG was dissolved in 0.2 M Tris-maleate buffer pH 6.4. After NTG treatment, the bacteria were washed again with sterile normal saline for 3 times and then inoculated on 2% skim milk heart-infusion agar. The caseinase negative colonies were selected and reinoculated on the skim milk medium to confirm the caseinase-deficient mutants.

Method for serological typing

P. aeruginosa antisera (Denka Seiken Co. LTD. Japan) was used. Serological procedure should be applied using slide agglutination test.

Test of pathogenicity to mice

The tested strains were cultured in HI broth (Difco) for 18 hrs. The supernatant was intravenously injected into mice's tail and numbers of death animals within a week were recorded. The bacteria pellet was washed with sterile normal saline for 3 times and suspension with different concentrations was made to challenge mice intravenously and intraperitoneally. LD_{50} was calculated by Reed-Muench method[23].

RESULTS

The distribution of enzymes involving

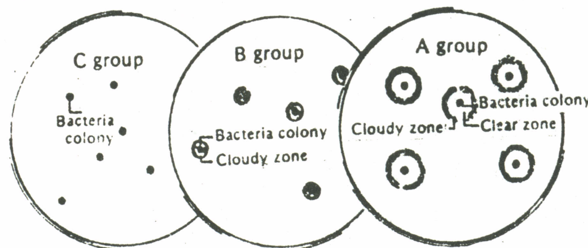

Fig. 1. Diagram illustrating caseinolytic activity of Pseudomonas aeruginosa reacting differently on skim milk HI agar plate. Group A makes a clear zone of complete hydrolysis around the colony and a cloudy precipitate zone around it; Group B forms a cloudy zone around the colony and Group C has no observable enzymatic activity.

Table 1. Distribution of *Pseudomonas aeruginosa* with Caseinolytic Activity on 2% Skim Milk Heart-Infusion Agar

Group*	Caseinolytic activity Strains (%)	Other extracellular enzyme activity (%) Lecithinase	DNase	Elastase
A	35 (72.9)	28 (80.0)	26 (74.3)	20 (57.1)
B	10 (20.8)	3 (30.0)	6 (60.0)	4 (40.0)
C	3 (6.3)	1 (33.3)	1 (33.3)	2 (66.7)
Tested strains	48 (100.0)	32 (66.7)	33 (68.8)	26 (54.2)

* Caseinolytic activity of ***Pseudomonas aeruginosa*** reacting differently on 2% skim milk HI agar plate. Type A makes a clear zone of complete hydrolysis around the colony and a cloudy precipitate zone around it; Type B forms a cloudy zone around the colony and Type C has no observable enzymatic activity.

Table 2. The Serological Type of *Pseudomonas aeruginosa*

Caseinase activity	Group (%) A	B	C	D	E	F	G	H	I	J	K	L	M	N	Untypable
A group (n=35)	0	1 (2.9)	1 (2.9)	0	6 (17.1)	2 (5.7)	4 (11.4)	1 (2.9)	2 (5.7)	0	2 (5.7)	3 (8.6)	1 (2.9)	0	12 (34.3)
B group (n=10)	0	1 (10.0)	0	1 (10.0)	3 (30.0)	0	0	0	2 (20.0)	0	0	0	0	0	3 (30.0)
C group (n=3)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3 (100.0)
Total (N=48)	0	2 (4.2)	1 (2.1)	1 (2.1)	9 (18.1)	2 (4.2)	4 (8.3)	1 (2.1)	4 (8.3)	0	2 (2.1)	3 (6.3)	1 (2.1)	0	18 (37.5)

Pseudomonas aeruginosa antisera (Denka Seiken Co. LTD. Japan) was used. Serological procedure should be applied using slide agglutination test.

caseinase, lecithinase, DNase and elastase in *Pseudomonas aeruginosa* was showed in Table 1. According to the presence of caseinolytic activity, *P. aeruginosa* was classified into three groups (Fig. 1). The A group made a clear zone of complete hydrolysis around the colony and a cloudy precipitate halo immediately surrounding colonies on skim milk HI agar. The B group had only cloudy zones surrounding colonies and the C group did not have caseinolytic activitv. The 72.9% of *P. aeruginosa* was mainly in A group. The ratios of B and C groups were 20.8% and 6.3%, respectively. The activity of lecithinase, DNase and elastase were higher in A group. All strains of clinically isolated *P. aeruginosa* had pyocyanin producing ability. Therefore this ability had no relationship with toxicity.

The serological typing of *P. aeruginosa* (Table 2)

After sero-typing by Denka Seiken *P. aeruginosa* antisera, 30 strains (62.5%) were typable and 18 strains were untypable (37.5%). Typable strains mainly distributed in E (18.1%), G (8.3%) and I groups (8.3%), A, J, and N groups were not identified. According to caseinase activity, the distributions of sera-typable and untypable strains

in A and B groups were very similar, but all untypable strains were in C group. The relationship of serological typing and elastase was shown in Table 3. The per cent of elastase positive strains in A, B and C groups were 57.1, 40.0 and 66.7.

The relationship between caseinase activity and toxicity to mice

The toxicity of washed bacteria cells suspension was shown in Table 4. The mice LD_{50} of caseinase A group distributed between 0.28 mg/kg and 11.23 mg/kg. In B group, the LD_{50} was from 1.15 mg/kg to 4.48 mg/kg. The pathogenicity was the weakest in C group, its LD_{50} was from 71.29 mg/kg to 103.75 mg/kg. It seemed that the extracellular substances did not influence *P. aeruginosa* pathogenicity to

Table 3. The Relation of Elastase Production to Serological Typing in *Pseudomonas aeruginosa*

Caseinolytic activity (group)	Serological typing		Total strains
	Typable	Untypable	
A	12/23 (52.2)#	8/12 (66.7)	20/35 (57.1)
B	2/7 (28.6)	2/3 (66.7)	4/10 (40.0)
C	0	2/3 (66.7)	2/3 (66.7)
Total strains	14/30 (46.7)	12/18 (66.7)	26/48 (54.2)

#The denominator indicates the number of tested strains; the numerator the number of elastase producing strains; figures in parentheses show the percentage.

Table 4. Virulence of *Pseudomonas aeruginosa* to ICR Mice with Intravenous Injection According to Caseinolytic Activity on 2% Skim Milk Heart-Infusion Agar

Dose administered (mg/kg; iv)	Mortality (%)* Caseinolytic activity**							
	A group				B group		C group	
	L(+)+ D(−) E(+) S(E)	L(+) D(+) E(+) S(I)	L(−) D(+) E(+) S(E)	L(−) D(+) E(−) S(E)	L(−) D(+) E(−) S(E)	L(+) D(+) E(−) S(L)	L(+) D(+) E(+) S(Un)	L(−) S(Un) E(−) S(Un)
160.00							100	100
80.00							20	60
40.00	100	100	100	100	100	100	0	0
20.00	100	100	100	100	100	100	0	0
10.00	100	100	80	40	100	80	0	0
5.00	100	100	60	0	100	60	0	0
2.50	100	100	0	0	100	20		
1.25	100	80	0	0	80	0		
0.63	80	20	0	0	20	0		
0.31	60	0	0	0	0	0		
0.16	20	0	0	0	0	0		
0.08	0	0	0	0	0	0		
LD_{50} (mg/kg)	0.28	0.88	5.00	11.23	1.15	4.48	103.75	71.27

*The results were observed for a week. Each group consisted of 5 ICR mice (♀; 16-20 gm body weight). LD_{50} was calculated with Reed-Muench method.

**Types of caseinolytic activity see table 1 foot notes.

+Abbreviation: L: Lecithinase production; D: DNase production; E: Elastase production and S: Serological typing (0-ag). +: -: Production ability (positive and negative).

mice. In caseinase A group, the iv LD_{50} was 0.88 mg/kg in lecithinase, DNase and elastase positive strain, the LD_{50} was 5.00 mg/kg, 0.28 mg/kg in only lecithinase positive strain, or DNase negative strain and the LD_{50} was 11.23 mg/kg in both lecithinase and DNase negative strains. The caseinase B group had the similar phenomenon. In C group, the LD_{50} of lecithinase, DNase and elastase all positive or all negative were 103.75 mg/kg and 71.27 mg/kg, respectively. Table 4 showed that the production abilities of lecithinase, DNase and elastase were not parallel to their pathogenicity to mice, but the caseinase activity was closely related to their pathogenicity. The LD_{50} of sera untypable strain (0.76 mg/kg; the result was not shown in table) had the similar result with typable strains (LD_{50}: 0.28 mg/kg - 11.23 mg/kg) in group A, so the serological typing and the pathogenicity did not have parallel relationship. The pyocyanin pigmentation also did not relate to their pathogenicity.

The toxicity of culture supernatant to mice (Table 5)

18 hrs HI broth culture was filtrated through millipore membrance and iv injected into mice (20 ml/kg). The mortality of mice was from 60.0% to 100% in caseinase groups A and B. The mortality was zero in C group. So, if the washed bacterial cell suspensions were virulent to mice, their supernatant also had toxicity. The exotoxin producing ability may be a very important pathogenic factor in *P. aeruginosa.*

The isolation and biologic characteristics of the caseinase-deficient mutants

2650 colonies were selected from 2% skim milk HI plates, and four caseinase-deficient mutants were obtained. These mutants were characterized by growth in NAC agar plates, 42°C incubation, serological typing and other biochemical tests. Moreover, the mutants were tested for the production of DNase, lecithinase, elastase and pyocyanin. The mutants had almost the same biologic characteristics as the parental strain except the ability to produce caseinase, elastase and pyocyanin (Table 6). These mutants also showed the same antigenicity with slide agglutination method.

Virulence of *P. aeruginosa* parental strain-113 and mutant-1 and -4

For determination of the LD_{50},

Table 5. Toxicity of Culture Supernatant of *Pseudomonas aerugonosa* to ICR Mice with Intravenous Injection

Group*	Enzyme activity: Lecithinase	DNase	Elastase	Mortality (%) (20 ml/kg; IV)
A	+	+	+	100.0
	+	—	+	100.0
	—	+	—	60.0
	—	+	+	80.0
B	+	+	—	60.0
	—	+	—	100.0
C	+	+	+	0
	—	—	—	0

The results were observed for 1 weeks. Each group consisted of 5 mice (ICR strain; ♀; 16-20 gm).
*Types of caseinolytic activity see Table 1 foot notes.

Table 6. Biological Characteristics of *Pseudomonas aerugonosa*-113 and Mutant Strains

Tested strains	Lecithinase	DNase	Caseinase	Pyocyanin	Elastase	Serological typing
Ps.-113 (parental)	+++	+++	+++	+++	+++	I group
Mutant-1	+++	+++	+	–	–	I group
Mutant-4	+++	+++	–	–	+	I group

Table 7. Mortality among Mice Used in Tests for Determination of the LD_{50} of *Pseudomonas aeruginosa* Parental Strain 113 and Mutant Strains when Administered IV and IP

Infecting route, dose administered (mg/kg)	Mortality (%)#		
	Parental strain	Mutant-1	Mutant-4
IV	(LD_{50} : 0.88mg/kg)	(LD_{50} : 8.02mg/kg)	(LD_{50} : 89.80mg/kg)
80.00		100	100
40.00	100	100	40
20.00	100	100	0
10.00	100	60	0
5.00	100	20	0
2.50	100	0	
1.25	80	0	
0.62	20	0	
0.31	0		
0.16	0		
IP	(LD_{50} : 0.73mg/kg)	(LD_{50} : 3.54mg/kg)	(LD_{50} : 71.27mg/kg)
80.00			100
40.00	100	100	60
20.00	100	100	0
10.00	100	100	0
5.00	100	80	0
2.50	80	20	0
1.25	60	0	
0.62	60	0	
0.31	20	0	
0.16	0	0	
0.08	0		

#The results of mortality were observed for a week. LD_{50} was calculated with Reed-Muench method. Each group consisted of 5 ICR mice (about 16-20 gm of body weight).

parental strain-113 and mutants were injected iv or ip into ICR mice weighing 16-20 gm (Table 7). The LD_{50} of wet washed cells suspension of parental strain-113 was 0.88 mg/kg by the iv route and 0.73 mg/kg by the ip route, the LD_{50} values of mutant-1 and -4 were compared with those of parental strain (Table 7). The virulence of the caseinase-deficient mutants was found to be much less than

Table 8. Toxicity of Culture Supernatant of *Pseudomonas aeruginosa* Parental Strain-113 and Mutants when Administered Intravenous Injection

Strains	18-hr HI broth culture supernatants		
	20 ml/kg	10 ml/kg	5 ml/kg
Parental strain-113	5/5 (100)#	5/5 (100)	2/5 (40)
Mutant-1	5/5 (100)	1/5 (20)	0/5 (0)
Mutant-4	0/5 (0)	0/5 (0)	0/5 (0)

#The denominator indicates the number of mice inoculated and the numerator is the number of mice died; figures in parentheses show the percentage.

that of parental strain. The LD_{50} of mutant-1 was 8.02 mg/kg by iv route and 3.54 mg/kg by ip route, but the LD_{50} was 89.80 mg/kg and 71.27 mg/kg respectively in mutant-4. These results also suggest that the caseinase production in *P. aeruginosa* is important in determining lethality to mice. Administration route may also affect the virulence of *P. aeruginosa* to mice.

Toxicity of culture supernatant of *P. aeruginosa* parental strain-113 and mutants to mice was showed in Table 8. The mice mortality for parental strain was 40.0% with 5 ml/kg of supernatant, mortality value for mutant-1 was 20.0% with 10 ml/kg of supernatant and for mutant-4 was 0% with 20 ml/kg of supernatant. Comparing the toxicity of parental strain-113 and mutants, we found the caseinase-deficient mutants were much less virulent than the parental strain. These results showed that the caseinase in *P. aeruginosa* was correlated with the virulence and exotoxin production.

DISCUSSION

For the isolation and identification of *Pseudomonas aeruginosa*, NAC and cetrimide agar were used as selective media; Neopeptone agar, Sabouraud maltose agar and Pseudomonas P & F agar were used for pigmentation identification. But for the determination of its pathogenicity or toxigenicity, there is still no proper method except animal experiment now. The serological typing and pyocine typing did not show any direct relationship with the virulence of *P. aeruginosa.* How can we find a quick and simple method to determine the virulence of clinically isolated *P. aeruginosa?*

The lecithinase production was often used to identify pathogenic bacteria, such as *Clostridium* and glucose non-fermentation gram-negative bacteria. Hugh and Gilardi[14] have reported that lecithinase production ratio in *P. aeruginosa* was 10.0%, *P. fluorescens* 92.0%. Tsai[20] used 10% EYGA medium and found that the lecithinase positive ratio of *P. aeruginosa* in Taiwan was 92.0%. We inoculated 48 clinically isolated strains on 10% EYGA medium and the lecithinase positive ratio was 64.6%, lower than Tsai' report. According to the classification of caseinase activity (Table 2), the caseinase A group had higher lecithinase positive ratio, 80.0%. But the

lecithinase production did not relate to the virulence of *P. aeruginosa.* For example, the bacteria cells iv LD_{50} of lecithinase, elastase and DNase positive strains in caseinase A group was 0.88 mg/kg; the mice mortality was 100.0% after 20 ml/kg iv injection with the culture supernatant. But for the lecithinase, elastase and DNase all positive strains in caseinase C group, the LD_{50} and mortality were 103.75 mg/kg and 0%, respectively. (Tables 4 and 5).

The measurement of elastase activity could be applied to identify the pathogenicity of *P. aeruginosa*[14-16]. Chen[24] had reported that the elastase positive ratios of clinically isolated strains and environmental strains were 87.2% and 85.7%. This time our result was only 54.2%, lower than Chen's report. According to the classification of caseinase activity, the elastase positive ratios of A, B, and C groups were 57.1%, 40.0% and 66.7%, respectively. From Table 5, we could also find that there was no direct relationship between the elastase production and the pathogenicity of *P. aeruginosa.* So we could not use the elastase production to evaluate the pathogenicity of *P. aeruginosa,* either.

The relations of DNase and pathogenicity had been reported by many bacteriologists, especially for *Staphylococcus aureus*[25]. We had found that Nissan's heart extract DNA medium could induce *P. aeruginosa* to produce DNase[21]. For the 48 clinically isolated strains, the DNase positive ratio of A, B and C groups was 74.3%, 60.0% and 33.3%, respectively. From Table 5, we could also find that DNase production could not be used to evaluate the pathogenicity of *P. aeruginosa.*

Hasegawa and Kondo[26] had compared the virulence of *Staphylococcus aureus* caseinase and bound coagulase-deficient mutants and parental strains. They thought that caseinase producing ability was related to the pathogenicity of *S. aureus.* The hydrolytic activity of *S. aureus* or *S. epidermidis* on skim milk medium had been used to identify their virulence[17]. By the caseinolytic activity on 2% skim milk HI agar, we classified *P. aeruginosa* into three groups: A group had a clear halo around the colony and a cloudy precipitate zone immediately around the clear halo; B group formed only a cloudy halo surrounding the colony and Group C had no observable enzymatic activity. In the 48 clinically isolated strains, 35 strains belonged to A group (72.9%), 10 strains belonged to B group (20.8%) and C group had only 3 strains (6.3%). As for their toxicity (Table 5), the bacterial cells and culture supernatant of A group showed the highest virulence, and the C group which did not have caseinase activity almost had no virulence. The caseinase was the extracellular product closely related to the pathogenicity and toxigenicity of *P. aeruginosa.*

Two major proteases have been isolated from culture fluids of *P. aeruginosa* [1,8]. Both of these enzymes have been implicated to be important virulence factors during colonization[1]. They contribute to the breakdown of physical barriers of the host, as well as enhancing bacterial proliferation by supplying amino acids and peptides from tissue proteins[27].

The results in animal experiments that have used purified proteases implicated them as being responsible for hemorrhages in internal organs[28], degradation of corneal proteoglycans[29], and increase in mortality [30]. Additionally, in vitro studies have shown degradation of fibrinogen[31], complement components[32], and immunoglobulins[33]. The pigment pyocyanin may contribute to the overall disease process through its effect on the oxygen up-take of tissue cells, including leukocytes[34].

The virulence of the caseinase mutants to mice was much weaker than that of parental strain-113. Mutant-1 lost pyocyanin and elastase producing ability while its caseinolytic activity was still kept but decreased clearly; Mutant-4 lost caseinase but possessed elastase activity (Table 6). Comparing their virulence (Tables 7 and 8), we found that the losing of elastase and pyocyanin production did not influence the pathogenicity and toxigenicity of *P. aeruginosa*. Ohman *et al.*[35-36] also showed that while exotoxin A was important in establishing infection, but elastase was not required. But the losing or decreasing of ability to elaborate caseinase activity resulted in concomitant loss of virulence of caseinase-deficient mutants. So the caseinase activity might be applied to identify the pathogenicity and toxigenicity of *P. aeruginosa.*

The infecting route of *P. aeruginosa* will influence their pathogenicity to mice. For parental strain-113 (Table 7), the iv LD_{50} was 0.88 mg/kg, but the ip LD_{50} was 0.73 mg/kg. For Mutant-1 and -4, the ip injection route also showed stronger infectivity. We know that the pathogenic factors of *P. aeruginosa* included exotoxins, proteolytic enzymes, leuckocidin, hemolytic substances, exoenzyme S, lipopolysaccharide, exopolysaccharide, and some cell surfaces components etc[1,5-13]. If *P. aeruginosa* was injected by iv route, the bacteria would be cleared out very soon by reticuloendothelial system (RES); while by ip injection, *P. aeruginosa* would have enough time to produce virulent factors mentioned above that protect the bacteria from leukocytosis and destruct or inhibit host's vital organs. So host's RES functions are very important against *P. aeruginosa* infection, especially for those compromised patients.

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以酪蛋白酶活性鑑定綠膿桿菌病原性之研究

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由臨床分離之 48 株綠膿桿菌依其對脫脂牛乳（skim milk）之反應分爲 3 群，卽A群產生完全分解的澄淸環及外圍以混濁之沉澱環（72.9%），B群產生混濁之沉澱環（20.8%）及C群之無酵素活性群（6.3%）等。

各種細菌經生理食鹽水淸洗之細菌懸浮液經靜脈注射後，其對 ICR 株小白鼠之LD_{50}分別是A群分佈於 0.28 mg／kg～11.23 mg／kg, B群 1.15 mg／kg～4.48 mg／kg，及C群 71.27 mg／kg～103.75 mg／kg（Table 4）。上淸液（20 mg／kg）靜脈注射後，A及B群之死亡率在 60-100%之間，而C群則無死亡（Table 5）。故酪蛋白分解活性群之綠膿桿菌具較強之病原性及產毒性。依血淸分型，A群可以分型者佔 65.7%，主要分佈於E及G群，但A、D、J及N則無菌株，而B群亦具類似之血淸型，C群3株全部屬非分型者，以彈力蛋白酶陽性率觀之，A群佔 57.1%，B群 40.0%及C群 66.7%，卵磷脂酶及去氧核醣核酸酶之陽性率分別是A群 80.0%及 74.3%，B群 30.0%及 60.0%，及C群 66.7%及 66.7%。

以N-methyl-N'-nitro-N-nitrosoguanidine 處理之酪蛋白酶缺乏變異株（caseinase-deficient mutants）與母株加以比較。其中變異株1號之生化特性之變化包括 pyocyanin及彈力蛋白酶產生能力之消失，及保持酪蛋白酶產生能力，變異株4號則消失酪蛋白酶及pyocyanin產生能力，但仍具彈力蛋白酶活性。由其細菌懸浮液對小白鼠之病原性加以分析，其靜脈注射及腹腔注射之LD_{50}分別是母株 0.88 mg／kg及 0.73 mg／kg，變異株1號是 8.02 mg／kg及 3.54 mg／kg及變異株4號 89.80mg／kg及 71.27 mg／kg；顯然注射途徑亦會影響綠膿桿菌對小白鼠之病原性。又以靜脈注射之LD_{50}母株較變異株1號約強 9.1倍及變異株4號強 102.0倍，以腹腔注射之病原性則相差 4.8倍及 97.6倍；由其培養上淸液靜脈注射後對小白鼠之毒性分別是母株以 5 ml／kg 注射時其死亡率是 40.0%，變異株1號以 10 ml／kg 注射之死亡率是 20.0%，而變異株4號以劑量高達 20 ml／kg 注射時仍無任何小白鼠死亡。

由以上之結果得知，綠膿桿菌之酪蛋白酶活性與其病原性及產毒性具非常密切的關係。而卵磷脂酶、彈力蛋白酶及去氧核醣核酸酶及pyocyanin等產生能力則和綠膿桿菌病原性並無直接關係，血淸分型亦無平行的關係。故行酪蛋白酶活性之測定是鑑定綠膿桿菌病原性及產毒性最佳指標之一。

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