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COMPARISON OF EXPERIMENTAL INFECTION IN MICE OF *CORYNEBACTERIUM RENALE* PILIATED AND NON-PILIATED CLONES

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Results of experimental infection in mice of *Corynebacterium renale* piliated and non-piliated clones were compared. No significant differences were found between the mice inoculated with piliated and non-piliated bacteria in the mortality, the number of mice positive for recovery of the bacteria from the urine, bladder and kidneys and the number of bacteria recovered from these urinary organs and tissues.

Key words: *Corynebacterium renale*, piliated clone, non-piliated clone, mice, infection

INTRODUCTION

C. renale is the causal agent of bovine pyelonephritis. It was the first gram-positive bacteria which were reported to possess pili.[16,17] Reports have been made of pili-mediated attachment of *C. renale in vitro* to trypsinized sheep erythrocytes[6], tissue culture cells[7], epithelial cells of the bovine urinary bladder[15] and attachment *in vivo* to the mucous membrane of the urinary bladder of mice.[8] But the role of pili in the pathogenesis of *C. renale* pyelonephritis is unknown. As a step tword elucidating this role, we compared the results of experimental infection in mice of *C. renale* piliated and non-piliated clones.

MATERIALS AND METHODS

Bacteria The piliated and non-piliated clones of *C. renale* strain No. 115 used in this study and the proportion of piliated bacteria in the population of each clone are listed in Table 1. P^+ and P^- clones were prepared previously from *C. renale* strain No. 115[12] and maintained in the authors' laboratory. P^+_{rc} and P^-_{rc} clones were recloned by the present authors from P^+ and P^-, respectively. The procedure of the recloning was as follows. One of the arbitrarily selected colonies of P^+ or P^- grown on nutrient agar was examined for piliation by the slide agglutination test with anti-pili

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TABLE 1 Proportion of piliated bacteria in each population of *C. renale* piliated and non-piliated clones.

Clone	Presence of pili[a]	Proportion of piliated bacteria	
p^+	+	196/200[b]	98.0%
p^-	−	2/350	0.6
p^+_{rc}	+	999/1000	99.9
p^-_{rc}	−	2/1000	0.2

[a] Determined by electron microscopy.
[b] piliated colonies were determined by the slide agglutination test with anti-pili antiserum. The numerator denotes the number of piliated colonies and the denominator denotes the number of colonies examined.

antiserum and by electron microscopy. One hundred colonies of the clone of P^+ or P^- were examined for piliation by the slide agglutination test with anti-pili antiserum and by electron microscopy. One of the arbitrarily selected P^+ or P^- clone, which was clonized twice, was examined again and designated as P^+_{rc} or P^-_{rc}. The proportion of piliated bacteria in each clone was examined with 200–1000 colonies of each clone by the slide agglutination test anti-pili antiserum. The results were 98.0% for P^+, 0.6% for P^-, 99.9% for P^+_{rc} and 0.2% for P^-_{rc}.

Anti-pili antiserum Anti-pili antiserum was prepared as previously reported.[6] Anti P^+ clone serum was absorbed with the bacteria of P^- clone. The bacteria of P^- clone collected from the culture grown on nutrient agar were mixed and incubated with anti P^+ clone serum at 37°c for 2 hours. The mixture was centrifuged and the supernatant was filtered through a membrane filter (Millipore Corp., 450nm). This procedure was repeated until the absorption was completed, which was confirmed by the slide agglutination test.

Medium and cultivation Bacteria were cultivated on nutrient agar (pH 7.2) at 37°c for 24 hours.

Mice Female ddY-F mice (22–30 g), 6 to 7 weeks old, were used. They were observed for a week before use.

Methods of inoculation Bacteria were suspended in phosphate-buffered saline (PBS, pH 7.4) and inoculated into mice, as previously described[13]. The mice were anesthetized with ether and their abdominal wall incised. A bacterial suspension of 0.025 ml containing approximately 10^6–10^8 colony forming units (CFU) was inoculated into the urinary bladder and then the abdominal incision was closed;.

Bacterial recovery Mice were forced to urinate by pressing lightly the abdomianl wall, and 0.2 ml of serial dilution of the urine was inoculated on nutrient agar daily. The mice died or were sacrificed from the 1st to 15th postinfection days and

examined shortly thereafter. The kidneys and bladder were removed aseptically and homogenized in PBS. Viable counts of 0.2 ml of serial dilution of kidneys and bladder homogenates were performed on nutrient agar. The number of recovered bacteria was expressed as $\log_{10}$ per kidney and bladder, or 0.02 ml of urine.

Serological examination 1) Anti-pili antibody response A serum sample was obtained from each mouse at sacrifice. The serum antibody titer against pili was determined by ELISA in each microplate using purified pili prepared as previously reported[11]. ELISA titers against pili were expressed as the reciprocal of the highest dilution showing an optical density (O.D.405nm) that was significantly higher than that of normal mouse serum by Smirnoff's rejection test[10] ($P<0.01$). Optical density (O.D.405nm) of normal mouse serum was examined as the negative control, and the mean and standard deviation (SD) of each normal serum titer ranged from 0.008 ± 0.001 to 0.054 ± 0.051. ELISA titers against pili of 18 normal mouse serum were less than 1:8. Considering these values and the high sensitivity of ELISA, titers 1:8–1:16 were not considered positive, and titers 1:32 or more were judged positive. 2) Anti P^+ or P^- bacteria antibody response The serum antibody titer against the inoculated bacteria was determined by the agglutination test using heat-killed (100°c, 30 min) P^+ or P^- bcteria prepared as previously reported[5]. A serial twofold serum dilution (0.05ml) was mixed with an equal volume of the antigen in wells of a microplate. The microplate was kept at room teperature overnight and the results were read. The titers were expressed as the reciprocal of the highest dilution showing definite agglutination. The agglutination titers adainst heat-killed P^+ or P^- bacteria of 18 normal mouse sera were less than 1:8, with a few mice showing an indefinite agglutination at 1:16. They were judged negative, and the antibody titer against the inoculated bacteria at 1:32 or more was judged positive.

Results

1) Comparison of mortality, number of mice positive for recovery of bacteria from the urine, bladder anad kidneys, anad the number of bacteria recovered from the kidneys and bladder

Mice were inoculated with approximately 10^6-10^8 CFU of piliated (P^+ or P^+_{rc}) or non-piliated (P^- or P^-_{rc}) bacteria. The mortality, number of mice positive for recovery of the bacteria from the urine, bladder anad kidneys, and the number of bacteria recovered from the kidneys anad bladder of the mice are shown in Table 2. No significant differences were found between the mice inoculated with piliated and non-piliated bcteria in the mortality and the number of mice positive for recovery of the bacteria from the urine, bladder and kidneys. There were no significant differences between the number of piliated and non-piliated bacteria recovered from the kidneys and the bladder. The only exception was the mice used in Experiment No. Ⅲ, which were inoculated with approximately 10^7 CFU of P^+ or P^- bacteria; in this

TABLE 2 Comparison of mortality, number of mice positive for recovery of bacteria from the urine, bladder and kidneys and number of bacteria recovered from the kidneys and bladder between the mice inoculated with piliated and non-piliated bacteria

Experiment No.	Clone	No. of bacteria inoculated (log_{10})	Days		Mortality	No. of mice positive for recovery of bacteria			No. of bacteria recovered from kidneys (log_{10})		No. of bacteria recovered from bladder (log_{10})	
			Mice that died	Mice Sacrificed		Urine	Bladder	Kidneys	Mice that died	Mice sacrificed	Mice that Died	Mice Sacrificed
I	p^+	6.3		14	0 / 5[a]	4 / 5	2 / 5	2 / 5		3.8±2.7[b](3)[c]		
	p^-	5.7		14	0 / 6	5 / 6	2 / 6	1 / 6		4.4(1)		
II	p^+	6.2		< 1	0 / 13		12 / 13	9 / 13		3.1±1.1 (13)		
	p^-	6.3		< 1	0 / 14		11 / 14	13 / 14		2.6±1.0 (18)		
III	p^+	7.2	5-12	15	5 / 6	5 / 6	5 1 6	5 / 6	6.1±1.0(9)*	0		
	p^-	7.0	3-11	15	4 / 6	5 / 6	4 / 6	4 / 6	4.9±1.1(7)	0		
IV	p^+	8.4	4,5	5	2 / 5	5 / 5	5 / 5	5 / 5	6.8±0.9(3)	4.4±0.7(3)		
	p^-	8.7	4,4	5	2 / 5	5 / 5	5 / 5	5 / 5	5.9±0.3(4)	4.6±0.4(3)		
V	p^+_{rc}	7.7	3	7	1 / 11	9 / 11	7 / 11	8 / 11	3.8(2)	3.4±0.8 (10)	6.7 (1)	4.2±2.1(6)
	p^-_{rc}	7.6		7	0 / 10	9 / 10	7 / 10	4 / 10		3.6±1.1(6)		4.0±1.7(7)
VI	p^+_{rc}	8.4	4,5	7	2 / 5	5 / 5	5 / 5	5 / 5	4.1±2.6(4)	3.0±1.8(2)	6.7±0.1 (2)	5.4±1.7(3)
	p^-_{rc}	8.3	7	7	1 / 5	5 / 5	5 / 5	5 / 5	3.2±1.0(2)	3.8±2.0(2)	6.8 (1)	6.0±0.6(4)

[a] The numerator denotes the number of mice positive and the denominator denotes the number of mice examined.
[b] Average of CFU and SD per kidney or bladder,
[c] parentheses denote the number of kidneys or bladders examined.
Blanks show not applicable or not tested.

Differences in the mortality and the number of mice positive for recovery of bacteria were evaluated by Fisher's exact probability test. Differences in the number of bacteria recovered from the kidneys and bladder were evaluated by student's t-test. No significant differences were found between the mice inoculated with piliated (p^+ or p^+_{rc}) and non-piliated (p^- or p^-_{rc}) bacteria in the mortality, the number of mice positive for recovery of bacteria from the urine, bladder and kidneys, and the number of bacteria recovered from the kidneys and bladder, except for the case marked with* ($p<0.05$).

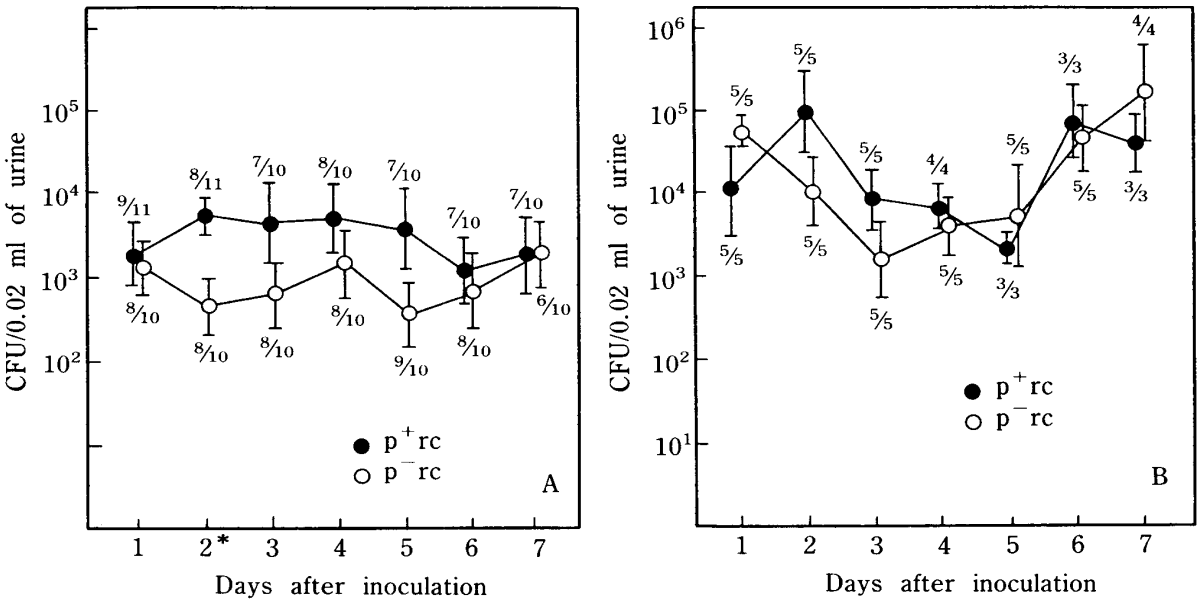

FIGURE. 1 Comparison of bacteria recovered from the urine of mice inoculated with p^+rc and p^-rc bacteria. A. Mice were inoculated with $10^{7.7}$ p^+rc or $10^{7.6}$ p^-rc bacteria (Experiment No. V). B. Mice were inoculated with $10^{8.4}$ p^+rc or $10^{8.3}$ p^-rc bacteria (Experiment No. VI). Fractions denote the number of mice that shed *C. renale* in the urine; the numerator denotes the number of mice positise for *C. renale* and the denominator denotes the number of mice examined. Average of CFU and standard error of p^+rc or p^-rc bacteria in the urine was shown. No significant differences in the number of bacteria recovered from the urine between the mice inoculated with p^+rc and p^-rcbacteria, except for the case marked* ($p<0.05$) by student's t-test.

case, more P^+ bacteria were recovered from the kidneys than P^- bacteria ($P<0.05$).

2) Comparison of the number of bacteria recovered from the urine of infected mice

The numbers of bacteria recovered daily from the urine of the mice inoculated with approximatly 10^7 and 10^8 CFU of P^+_{rc} or P^-_{rc} bacteria are shown, respectly, in Figures 1A and 1B. The number of mice positive for recovery of the bacteria was similar between the mice inoculated with P^+_{rc} and P^-_{rc} bacteria. There were no significant differences between the number of P^+_{rc} and P^-_{rc} bacteria shed in the urine. The only exception was noted on the 2nd day in the mice inoculated with approximately 10^7 CFU of bacteria; in this case, P^+_{rc} bacteria were recovered in significantly greater numbers than P^-_{rc} bacteria ($P<0.025$).

TABLE 3 Anti-pili ELISA titer and anti-p^+ or p^- heat-killed bacteria agglutination titer in the serum of mice inoculated with piliated (p^+ or p^+_{rc}) and (p^- or p^-_{rc}) bacteria

Clone	No. of bacteria inoculated (log_{10})	Days mice were sacrificed	Anti-pili ELISA titer	Anti-p^+ or p^- bacteria agglutination titer
p^+	6.3	14	2048(1)[a]	512(1)
		14	1024(1)	64(1)
		14	512(2)	32(1)
		14	< 8(1)	16(1)
		14		< 8(1)
			32(1)	
	7.2	15		< 8(1)
			128(1)	
	8.4	5	8(1)	<40(1)
		5	< 8(1)	< 8(2)
		5		
			64(1)	32(1)
p^+_{rc}	7.7	7	32(1)	8(2)
		7	8(1)	< 8(7)
		7	< 8(7)	
		7		
			256(1)	8(2)
	8.4	7	128(1)	< 8(1)
		7	< 8(1)	
p^-	5.7	14	16(1)	16(1)
		14	< 8(5)	< 8(5)
	7.0	15	< 8(2)	< 8(2)
	8.7	5	< 8(3)	< 8(3)
p^-_{rc}	7.6	7	< 8(10)	< 8(10)
	8.3	7	16(1)	16(1)
			< 8(3)	< 8(3)
Control[b]			< 8(18)	< 8(18)

[a] parenthesis shows number of mice.
[b] Normal mouse serum

3) Serological response of mice

The serological reponse of the mice inoculated with piliated (P^+ or P^+_{rc})and non-piliated (P^- or P^-_{rc}) bacteria and sacrificed from the 5th to 15th postinfection days were examined by ELISA and the agglutination test (Table 3).

The antibody response against pili in the mice inoculated with piliated bacteria (P^+ or P^+_{rc}) was positive in nearly half of the mice. In the mice inoculated with non-piliated bacteria (P^- or P^-_{rc}), on the other hand, the antibody response against pili was not positive.

The antibody response against heat-killed P^+ bacteria in the mice inoculated with piliated bacteria (P^+ or P^+_{rc}) was positive in 4 of 22 mice. On the contrary, in the mice inoculated with non-piliated bacteria (P^- or P^-_{rc}), the antibody response against heat-killed P^- bacteria was not positive.

Discussion

Pili is believed to mediate bacterial adherence, which is considered to be a prerequisite for urinary tract infection. In human urinary pathogens such as *Escherichia coli*,*Proteus mirabilis* and *Klebsiella pneumoniae*, it was reported that piliated bacteria have more infectivity compared with non-piliated bacteria in mouse or rat models.[1,2,9,14] In the mouse model of *C. renale* pyelonephritis shown in the present study, there were no significant differences between the mice inoculated with piliated and non-piliated bacteria in mortality, the number of mice positive for recovery of bacteria from the urine, bladder and kidneys, and the number of bacteria recovered from these urinary organs and tissues. The present results may indicate that piliation is not always advantageous to *C. renale*.

The conditions in the present study were as follows. Approximately 10^6–10^8 CFU of *C. renale* pliated or non-piliated bacteria were inoculated into the urinary bladder of mice and the mice were sacrificed from the 1st to 15th postinfection days. This condition may not be advantageous to piliated bacteria. Piated bacteria gain an advantage by becoming attached to portals of entry such as bovine vulva epithelial cells with pili.[3,4] In the present study, such attachment of the first stage of natural infection of *C. renale* was bypassed, because piliated or non-piliated bacteria were inoculated directly into the urinary bladder to bring about a more advanced stage of *C. renale* infection. An attempt to produce retrograde pyelonephritis in mice by inoculating *C. renale* bacteria into the vagina was not successful. The establishing such an animal model inovolving the first stage of natural infection needs further study.

Anti-pili antibody was detectable in the serum of nearly half of the mice inoculated with piliated bacteria. The effect of anti-pili antibody on the piliated bacteria of *C. renale* is not known *in vivo* and requires further investigation.

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