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Study on pathogenesis of *Enterococcus faecalis* in urinary tract

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Summary. The rate of isolation of *Enterococcus faecalis* as the causative bacterium of complicated urinary tract infections has been increasing. However, the pathogenicity of this bacterium in the urinary tract has not been clarified. Thus, the authors investigated how *E. faecalis* is pathogenic to the urinary tract, using mice with experimental urinary tract infection. The investigation revealed that this bacterium when sufficiently numerous can be directly pathogenic. The bacterium can be pathogenic indirectly when present with other typical urinary bacteria such as *E. coli*.

Key words: *Enterococcus faecalis* – Urinary tract infection – Pathogenicity

Introduction

Enterococcus faecalis, is resistant to Cephalosporins and has increasingly been isolated due to the phenomenon of bacterial replacement. In urinary tract infection, the incidence of isolation of *E. faecalis* has been increasing, and this has become a clinical problem [7, 9]. Little has been known about the degree of pathogenicity of this bacterium. In order to clarify these issues, we conducted basic studies on (1) the ability of this bacterium to adhere to epithelial cells exfoliated from the urinary tract and (2) the capacity of this bacterium to cause pyelonephritis in mice by experimental inoculation into the urinary bladder.

Materials and methods

In vitro adhesion assay

The in vitro adhesion assay method of Svanborg-Eden [10] was employed with modifications.

E. faecalis (6017 gamma, 605 gamma and 59147), *E. faecium* (6032 alpha), *P. aeruginosa* (0G) and *E. coli* (06) were isolated from patients with urinary tract infections. Each isolate was suspended in pH 7.3 phosphate buffered saline (PBS) at a cell density of 10^6 cfu/ml. Epithelial cells exfoliated from the urinary tract (exfoliated uroepithelial cells) were collected from freshly-voided midstream urine of 5 healthy adult females (age: 34–50 years) and suspended in PBS at a cell density of 10^4 epithelial cells/ml. A 0.5-ml aliquot of each bacterial suspension was mixed with a 0.5-ml aliquot of the epithelial cell suspension, and the mixture was incubated at 37°C for one h. The number of bacterial cells adhered to 40 epithelial cells was counted. The mean number of bacterial cells adhered per epithelial cell was used as the index of the adhesive capacity of the bacterium.

Virulence of E. faecalis to cause pyelonephritis

A 0.05 ml-aliquot of each of three *E. faecalis* (6017 gamma) cell suspensions (cell density: 10^5 cfu/ml, 10^7 cfu/ml and 10^9 cfu/ml) was transurethrally injected into the urinary bladder of 6-week-old ddY female mice (18–27 mice for each bacterial suspension). After the injection, the urethra was obstructed with a clamp for 2 h. The kidney were then isolated 6 and 24 h and 3 and 7 days after the injection, fixed with a pH 7.3 periodate lysine-paraformaldehyde (PLP) solution and embedded in paraffin [4]. Each section of the specimens was subjected to hematoxylineosin staining and observed under a microscope. The degree of infiltration of the tissues by leukocytes was used as the index of the degree of inflammation. The severity of pyelonephritis was rated by the following 4 grades. (+++): when infiltration by leukocytes extended to the surface of the kidney; (++): when it extended to the cortex; (+): when it extended to the medulla or the periphery of the pelvis; and (−): when no infiltration by leukocytes was observed.

Counting of bacterial cells in bladder urine and kidney

The urine in the bladder and the kidney were aseptically collected or isolated, and their weights were measured. The isolated kidney was homogenized in 5 ml of PBS solution. Each of the urine and the kidney homogenates was appropriatly diluted, and each of the diluted samples was spread on top of a heart infusion agar plate (Difco). After incubation, the number of bacterial colonies formed on the plate was counted. In the case of *P. aeruginosa*, nalidixic acid-cetrimide (NAC) agar plates (Eiken) were used for colony counting.

Table 1. Adhesion of urinary isolates to uroepithelial cells

Bacterium	cfu per epithelial cell[a]
E. faecalis (6017 γ)	23.7 ± 11.3*
E. faecalis (605 γ)	7.9 ± 3.0
E. faecalis (59147)	20.8 ± 5.4
E. faecium (6032 α)	24.1 ± 10.3
P. aeruginosa (0G)	40.1 ± 14.3**
E. coli (06)	40.6 ± 23.1***

[a] Exofoliative cells from five healthy females. Mean ± SD(calculated by counting bacteria adhered to 40 exfoliative cells)
* vs ** $0.10 > P > 0.05$
* vs *** $0.20 > P > 0.10$

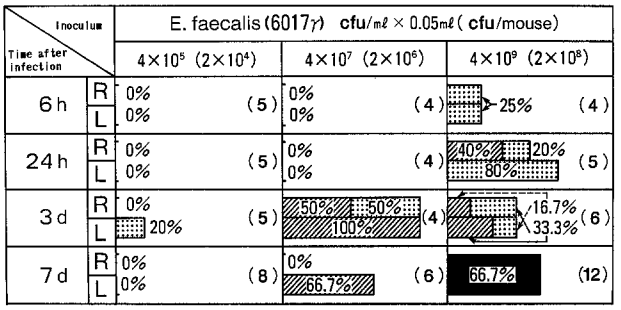

Fig. 1. Experimenta pyelonephritis in mice transurethrally infected with *Enterococcus faecalis*. Degree of inflammation: ■ = +++; ▨ = ++; ▩ = +. R = right kidney; L = left kidney; number in brackets = *N*

Statistical method

All the data generated from the present experiments were tested for significant differences by unpaired Student's *t* test.

Results

Adhesion of E. faecalis to exfoliated uroepithelial cells

E. faecalis (6017 gamma, 605 gamma, 59147), *E. faecium* (6032 alpha), *P. aeruginosa* (0G) and *E. coli* (06) were compared for their ability to adhere to the exfoliated uroepithelial cells. Although no significant difference was detected among these bacteria, there was a tendency for *E. faecalis* (6017 gamma) to show less adhesion than *E. coli* and *P. aeruginosa* (Table 1).

Virulence of E. faecalis to cause pyelonephritis in mice after injection into urinary bladder

Mice were experimentally infected with *E. faecalis* (6017 gamma) alone by injecting a cell suspension having a cell density of 10^5, 10^7 or 10^9 cfu/ml into the urinary bladder. In the animal group infected with the bacterium at a cell density of 10^5 cfu/ml, almost no mice developed pyelonephritis. However, in both the other two animal groups (infected at 10^7 and 10^9 cfu/ml), pyelonephritis was detected in 50–100% of the animals 3 and 7 days after the experimental infection (Fig. 1.). Thus, it was found that *E. faecalis* (6017 gamma) is sufficiently virulent to cause pyelonephritis when 0.05 ml of a suspension of this bacterium with a cell density of 10^7 cfu/ml or higher is injected into the urinary bladder of mice.

Experimental mixed infection was established using *E. faecalis* and *E. coli*. To determine the appropriate cell density of *E. coli* (06) for this mixed infection study, this bacterial strain was injected alone to the urinary bladder using 0.05 ml each of 10^5 cfu/ml and 10^3 cfu/ml cell suspension. Pyelonephritis was detected 3 and 7 days after inoculation in 90–100% of the mice infected with the 10^5 cfu/ml cell suspension, while this disease was detected in less than 10% of the mice infected with the 10^3 cfu/ml suspension. Thus, a mixed infection experiment was conducted employing a 10^3 cfu/ml *E. coli* (06) suspension – which does not cause pyelonephritis when injected alone – and 10^3, 10^5 and 10^7 cfu/ml suspensions of *E. faecalis* (6017 gamma). In this experiment, to clearly express the degree of inflammation, a scoring method was employed. That is, a score of "3" was given if a mouse died due to urosepsis or the severity of pyelonephritis was rated as (+++), "2" if the rating was (++) and "1" if the rating was (+). The mean (for the group) ± S.D. of a score was used as the index of the virulence to cause pyelonephritis.

Pyelonephritis, as evaluated 3 and 7 days after the inoculation, was clearly intensified by the mixed infection consisting of simultaneous injection of a 10^5 cfu/ml *E. faecalis* (6017 gamma) suspension and a 10^3 cfu/ml *E. coli* (06) suspension (only 200 cfu of *E. coli*/mouse). At these inocula, neither of these bacteria is capable of causing pyelonephritis when injected singly. However, when a 10^7 cfu/ml *E. faecalis* suspension was injected together with a 10^3 cfu/ml *E. coli* suspension, the pyelonephritis was not intensified. It was surmised that since *E. faecalis* is sufficiently virulent to cause pyelonephritis alone at a cell density of 10^7 cfu/ml, the effect of a small amount of *E. coli* might have been too little to detect (Table 2).

Bacterial count in kidney and bladder urine in mixed infection

To investigate the reasons for the increase in the virulence to cause pyelonephritis in the mixed infection, the number of bacteria in each of the kidney and bladder urines was counted. First, mice were subjected

Table 2. Score of virulence to cause experimental pyelonephritis in mice ($N=8\sim10$) with *E. faecalis* alone and *E. coli* mixed

	Time after injection	Score (mean ± SD)				
		Inoculum of *E. faecalis* (6017 γ) cfu/ml (×0.05 ml)				
		0	4×10^3	4×10^5	4×10^7	4×10^9
E. faecalis	6 h	-	-	0	0	0.3±0.5
(6017 γ)	1 day	-	-	0	0	0.9±0.7
single infection	3 days	-	-	0.1±0.3*	1.8±0.5	0.8±0.9
	7 days	-	-	0**	0.7±1.0	2.3±1.0
E. coli (06)	6 h	0	0	0	0	-
4×10^3/ml (×0.05 ml)	1 day	0	0	0.5±0.6	0.3±0.5	-
mixed infection	3 days	0.3±0.5	0.8±1.0	0.8±0.4*	0.3±0.5	-
with *E. faecalis*	7 days	0.4±0.7	0	1.5±1.2**	1.3±1.2	-

* $P<0.01$
** $P<0.05$

Table 3. Bacterial counts in kidney and bladder urine of mice with experimental single infection

	Bacterial count in kidney	% of mice				Bacterial count in bladder urine	% of mice			
		Time after infection					Time after infection			
		6 h	1 day	3 days	7 days		6 h	1 day	3 days	7 days
E. faecalis[a] (6017 γ)	$0\sim<4\times10^5$ cfu/g	100	100	100	100	$0\sim<4\times10^5$ cfu/ml	80	100	100	100
(10 mice)	$\geqq4\times10^5$ cfu/g	0	0	0	0	$\geqq4\times10^5$ cfu/ml	20	0	0	0
E. coli[a] (06)	$0\sim<4\times10^3$ cfu/g	100	100	50	75	$0\sim<4\times10^3$ cfu/ml	100	100	75	75
(12 mice)	$\geqq4\times10^3$ cfu/g	0	0	50	25	$\geqq4\times10^3$ cfu/ml	0	0	25	25

[a] single inoculum sizes: *E. faecalis*, 4×10^5 cfu/ml×0.05 ml ($=2\times10^4$ cfu/mouse); *E. coli*, 4×10^3 cfu/ml×0.05 ml ($=2\times10^2$ cfu/mouse)

to a single infection with a 10^5 cfu/ml *E. faecalis* (6017 gamma) suspension or a 10^3 cfu/ml *E. coli* (06) suspension, and the change in the count of each bacterium in the kidney and bladder urine was monitored with time. As a result, in almost none of the mice infected with *E. faecalis* alone were bacteria detected in the kidney or the bladder urine one or more days after the inoculation. In the case of *E. coli* single infection, as well, the bacterial counts in the kidney and the bladder urine were higher than the inoculum in only 25% of the mice 7 days after the inoculation (Table 3). On the other hand, in the mixed infection with the 10^5 cfu/ml *E. faecalis* (6017 gamma) suspension and the 10^3 cfu/ml *E. coli* (06) suspension, the bacterial counts of *E. faecalis* in the kidney and the bladder urine were higher than the inoculum in 67% of the mice 7 days after the inoculation. Regarding *E. coli*, the bacterial counts in the kidney and bladder urine were higher than the inoculum in 67% of more of the mice one or more days after the inoculation (Table 4). That is, in the mixed infection, each bacterial species was found to proliferate, and this was surmised to be the reason for the increased virulence.

Virulence of other bacterial species to cause pyelonephritis in mice after injection of bacteria into urinary bladder

Other clinical bacterial isolates were investigated for their virulence to cause pyelonephritis. No other strains of *E. faecalis* and no strains of *E. faecium* caused pyelonephritis in any mice singly infected with a 10^5 cfu/ml suspension of the respective strain. However, when each of these strains (at 10^5 cfu/ml) was injected together with a 10^3 cfu/ml *E. coli* (06) suspen-

Table 4. Bacterial counts in kidney and bladder urine of experimental mixed infection in mice ($N=12$)

	Bacterial count in kidney	% of mice				Bacterial count in bladder urine	% of mice			
		Time after infection					Time after infection			
		6 h	1 day	3 days	7 days		6 h	1 day	3 days	7 days
E. faecalis[a] (6017 γ)	$0 \sim < 4 \times 10^5$ cfu/g	100	67	75	33	$0 \sim < 4 \times 10^5$ cfu/ml	100	100	75	33
	$\geqq 4 \times 10^5$ cfu/g	0	33	25	67	$\geqq 4 \times 10^5$ cfu/ml	0	0	25	67
E. coli[a] (06)	$0 \sim < 4 \times 10^3$ cfu/g	100	33	0	33	$0 \sim < 4 \times 10^3$ cfu/ml	100	33	25	33
	$\geqq 4 \times 10^3$ cfu/g	0	67	100	67	$\geqq 4 \times 10^3$ cfu/ml	0	67	75	67

[a] mixed inoculum sizes: *E. faecalis* 4×10^5 cfu/ml and *E. coli* 4×10^3 cfu/ml of mixed bacterial suspension $\times$ 0.05 ml

Table 5. Score of virulence to cause experimental pyelonephritis in mice ($N=8 \sim 12$) with various single and *E. coli* mixed infections

Bacterium	Time after infection			
	Day 3		Day 7	
	Single infection[a]	Mixed infection[b]	Single infection[a]	Mixed infection[b]
E. faecalis (605 γ)	0.1 ± 0.4*	0.9 ± 0.6*	0*	1.4 ± 1.1*
E. faecalis (6065 β)	0.1 ± 0.4*	1.7 ± 0.8*	0.3 ± 0.5	0.9 ± 1.2
E. faecalis (SS498)	0*	0.8 ± 0.6*	0.2 ± 0.4**	1.4 ± 1.3**
E. faecium (6032 α)	0.3 ± 0.8	0.5 ± 0.7	0.1 ± 0.4	0.9 ± 1.1
E. faecium (6050 α)	0*	1.4 ± 1.1*	0.2 ± 0.4**	1.0 ± 0.9**
E. faecium (SS442)	0.1 ± 0.4	0.4 ± 0.5	0.2 ± 0.4	0.5 ± 0.7
S. saprophyticus (5947)	0.1 ± 0.4*	0.8 ± 0.6*	0.6 ± 0.5	0.9 ± 0.9
S. epidermidis (6045)	0.4 ± 0.7	0.9 ± 1.0	1.0 ± 1.2	1.6 ± 1.2
P. aeruginosa (OG)	1.2 ± 1.2	1.4 ± 1.2	2.3 ± 1.4	1.8 ± 1.4
E. coli (06)	2.1 ± 1.0	-	2.2 ± 1.1	-

[a] Inoculum size for single infection: 4×10^5 cfu/ml of each bacterium $\times$ 0.05 ml ($= 2 \times 10^4$ cfu/mouse)
[b] Inoculum size for mixed infection: 4×10^5 cfu/ml of each bacterium with E. coli (06) 4×10^3 cfu/ml $\times$ 0.05 ml
* $P < 0.01$
** $P < 0.05$

sion, they caused pyelonephritis as was observed with *E. faecalis* (6017 gamma). The severity of pyelonephritis caused by single infection with *S. saprophyticus* or *S. epidermidis* was somewhat higher than that caused by single infection with the species of *Enterococcus*, and the virulence of these species to cause pyelonephritis was increased when each was injected together with a 10^3 cfu/ml *E. coli* suspension. In the case of *P. aeruginosa*, its virulence to cause pyelonephritis was high when it was injected alone as a 10^5 cfu/ml suspension. Thus, when that suspension was injected together with a 10^3 cfu/ml *E. coli* suspension, the severity of pyelonephritis was not very greatly affected by the presence of *E. coli*. Injection of a 10^5 cfu/ml *E. coli* (06) suspension alone caused strong pyelonephritis in the mice (Table 5).

Discussion

In parallel with the increased clinical use of second- and third-generation cephalosporins in recent years, *E. faecalis*, whose susceptibility to those antibiotics is low, has come to be isolated more frequently [7, 9]. In particular, there is a problem of increased involvement of this bacterium in compromised hosts with complicated urinary tract infections [2]. In our Department, the incidence of isolation of *E. faecalis* from inpatients with urinary tract infections was only 6.2% in 1977, but it rapidly increased in the 1980s, becoming as high as 20% in 1987. Thus, *E. faecalis*, which in the past was considered to have low pathogenicity, is now isolated as a causative microbe from about 1/5 of patients with urinary tract infections. Therefore, we conducted a

basic investigation to elucidate the pathogenicity of *E. faecalis* for the urinary tract.

Adhesion of bacteria to uroepithelial cells is the crucial first step in the onset of urinary tract infections. Thus, we first compared various clinical isolates for the degree of adhesion to uroepithelial cells in vitro [5, 6, 10, 11]. In comparison with an adhesion value (the degree of adhesion to uroepithelial cells, expressed as the number of bacterial cells per uroepithelial cell) of 40 cfu observed with *E. coli* and *P. aeruginosa, E. faecalis* was found to have a smaller adhesion value of about 20 cfu. Accordingly, it was surmised that *E. faecalis* can be somewhat pathogenic for the urinary tract.

E. faecalis was investigated in vivo for its virulence in mice by means of experimental infection of the urinary bladder. As a result, considerable virulence was detected with *E. faecalis* when 0.05 ml of its cell suspension with a cell density of 10^7 cfu/ml or higher was injected alone. Miyazaki et al. [8] studied the pathogenicity of *E. faecalis* by investigating its virulence in causing systemic infection in mice, and they reported that beta-type hemolytic strains are more virulent than non-hemolytic strains. Since *E. faecalis* (6017 gamma) employed in the present study is a gamma-type strain (non-hemolytic strain), its virulence was anticipated to be weak, but the present study revealed that this strain has virulence to cause pyelonephritis when it is inoculated at a high cell density. *E. faecalis* is frequently isolated together with gram-negative rods as the causative bacteria of mixed infections [3]. Thus, with the purpose of elucidating the influence of this bacterium on the host and other causative bacteria in mixed infections, we conducted experimental mixed infection employing *E. coli* (06), which is a representative causative bacterium of pyelonephritis. When mice were simultaneously inoculated with a 10^5 cfu/ml *E. faecalis* suspension and a 10^3 cfu/ml *E. coli* suspension – cell densities at which neither strain is capable of causing pyelonephritis when given alone – pyelonephritis developed in 60–100% of the mice, indicating an increase in their virulence in combination. In this experiment, each bacterium was found to have proliferated in the kidney and bladder urine, and this fact was surmised to be the reason for the increased virulence. Thus, from the viewpoint of *E. coli* infection at a small inoculum size, concurrent infection with *E. faecalis* at a moderate inoculum size (about 10^5 cfu/ml) might lower the host's self-defense mechanism against infections as a result of invasion of the urinary tract epithelium by *E. faecalis,* leading to proliferation of sparse *E. coli* and eventual establishment of a pyelonephritis by the proliferated *E. coli.*

Infection with *E. faecalis* at a moderate inoculum size is might indirectly enhance the proliferation of gram-negative rods such as *E. coli* which are present in the host at a low cell density. This growth-enhancing effect of *E. faecalis* was also seen to occur for other gram-positive cocci and gram-negative rods. Accordingly, it is thought that the presence of *E. faecalis* in mixed infections of the urinary tract cannot be ignored.

This basic study on *E. faecalis* revealed that this bacterial species is less pathogenic than *E. coli* and *P. aeruginosa*, but that even alone it can cause pyelonephritis if it infects the host at a high density. In addition, infection of the host by *E. faecalis* at a moderate density (ca. 10^5 cfu/ml) may enhance the onset of urinary tract infections by other causative bacteria such as gram-negative rods. Therefore, if *E. faecalis* is isolated at a cell density of 10^5 cfu/ml or higher from the urine of a host compromised due to aging, etc., the bacterium sometimes could cause urosepsis in the host [1]. The present study thus indicates *E. faecalis* has direct or indirect pathogenicity to the urinary tract which is lower than *E. coli* or *P. aeruginosa.*

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