Establishment of Specific Pathogen-Free (SPF) Rat Colonies using Gnotobiotic Techniques

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Abstract: Gnotobiotic Wistar rats were produced using gnotobiotic techniques, which were established in the production of a SPF mouse colony, in order to establish a barrier-sustained colony. One strain of *Escherichia coli*, 28 strains of *Bacteroidaceae* (B-strains), three strains of *Lactobacillus* (L-strains) and a chloroform-treated fecal suspension (CHF, *Clostridium* mixture) were prepared from conventional Wistar rats as the microflora source. Two groups of limited-flora rats, *E. coli* plus B-strains and *E. coli* plus CHF, were produced. After confirmation that *Clostridium difficile* was not detected in the CHF-inoculated rats, two groups of limited-flora rats were transferred to an isolator and housed together in a cage. These rats were then orally inoculated with L-strains. The gnotobiotic rats showed colonization resistance to *Pseudomonas aeruginosa*, and the number of *E. coli* in the feces was 10^5 to 10^6 /g. The gnotobiotic rats were transferred to a barrier room as a source of intestinal flora for SPF colonies. In the SPF rats, basic cecal flora was mainly composed of *Bacteroidaceae*, clostridia, fusiform-shaped bacteria and lactobacilli, and did not change over a long period. Their flora became similar to that of conventional rats.

Key words: cecal microflora, gnotobiote, limited-flora rats, normalization, SPF rats

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Introduction

Specific pathogen-free (SPF) animals are generally produced using hysterectomy-derived animals and maintained in barrier type animal houses. However, the barrier system eliminates and prevents not only infectious agents but also floral organisms. Consequently, SPF animals lack the indigenous flora that resist pathogenic species or opportunistic pathogens. Moreover,

SPF animals are produced with different combinations of intestinal bacteria and maintained in different environments [4]. These variations in the bacteriological status of animals can influence experimental results [18]. Therefore, the production of gnotobiotic animals with colonization resistance to opportunistic infection and normal physiological function is required for the establishment of SPF colonies [1, 3, 19].

We attempted to produce SPF rat colonies utilizing

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gnotobiotic techniques reported by Itoh *et al.* [5, 7–9, 11, 12], and monitored the status of cecal flora and pathogenic microbes in the SPF rats.

Materials and Methods

Animals: Three conventional (CV) male Wistar rats (10 weeks of age) obtained from a commercial supplier (Ishikawa Farm, Saitama, Japan) were used as donor rats for intestinal flora. Sendai virus, Hantavirus, *Mycoplasma* spp. and *Pseudomonas aeruginosa* were not detected in the CV rats. A total of 24 recipient germfree (GF) rats (male: 8, female: 16) were derived by hysterectomy from Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) and raised under axenic conditions in flexible vinyl isolators. All animals were given 50 kGy γ -irradiated diet (MR-A1, Nihon Nosan, Yokohama, Japan) *ad libitum*. SPF Wistar rats (Japan SLC, Inc.) were used as existing SPF rats. The CV and SPF rat colonies were established about 15 and 20 years ago, respectively.

Microorganisms: Five strains of *Escherichia coli* were isolated from the cecum of CV rats using DHL agar plates, and one of the strains was selected based on typical colony morphology and biochemical characteristics. Forty Bacteroidaceae strains in total were isolated from the cecum using NBGT agar plates (steel wool jar method), SM10 and EG agar plates (anaerobic chamber) according to the methods described earlier [9]. The isolates were divided into 28 types (B-strains) on the basis of colony morphology, Gram staining, cell morphology, and aerotolerance. Ninety isolates of *Lactobacillus* from the stomach, small intestine, and cecum were characterized on the basis of cell and colony morphology, acid production from carbo-hydrates, and gas formation from glucose by the methods described earlier [7]. *L. acidophilus* II a, *L. murini* II and *L. reuteri* I d, which are predominant strains in rat intestines, were used as rat *Lactobacillus* (L-strains). A chloroform-treated cecal suspension (CHF) [5] was used as rat *Clostridium*. Confirmation of no contamination with *C. difficile* in CHF was performed by the methods described previously [10].

Production of limited-flora (LF) rats: Bacteroidaceae and *Clostridium* LF rats were produced by inoculation with *E. coli* plus B-strains and *E. coli* plus CHF, respectively.

Production of gnotobiotic (GB) rats: Two *Clostridium* LF rats were transferred to a Bacteroidaceae LF rat isolator and inoculated with three *Lactobacillus* strains.

Colonization resistance to *P. aeruginosa*: Three months after production of GB rats, four GB rats, 8–10 weeks of age, were transferred to a germfree isolator and were given HCl-supplemented water (pH 2.7–3.0). On the following day, *P. aeruginosa* strain 15 [9] was inoculated into the stomach of GB rats at a concentration of 10^5 cells/0.2 ml. Two and 3 weeks after inoculation, drinking water, feces and mucus membrane from the pharynx were inoculated in nalidixic acid-cetrimide (NAC) broth and incubated at 37°C for 48 hr. One loopful of the growth was placed on NAC agar and incubated at 37°C for 48 hr in order to confirm pigment production [16].

Cecal size: Cecae were weighed and relative weight was expressed as a percentage of body weight. Determinations of cecal size were made when cecal flora of GB rats was examined.

Maintenance of gnotobiotic rats: The GB rats were kept in small and large flexible vinyl isolators (small; 650 × 650 × 1250 mm, large; 900 × 1700 × 1700 mm) in the same way as GF animals, and autoclaved water and diet (MR-A1 for breeding, MR-A2 for maintenance; Nihon Nosan, Yokohama, Japan) were given *ad libitum*. Seven GF rats were transferred into the GB isolator one month after production of GB rats. After colonizing the GF rats with the intestinal flora of GB rats, GB and new GB rats were bred in an isolator.

Transfer of GB rats to barrier rooms: GF pups removed by hysterectomy were fostered by a GB lactating mother to transplant the intestinal flora of GB rats to them. During six months, pups from more than 70 pregnant rats were fostered. A 5 to 6 weeks of age, they were transferred from the isolators to the existing barrier room. Some of the GB rats were maintained in the isolators to retain their flora.

Maintenance of SPF rats: The animals were kept in the barrier unit, in which the existing SPF rats mentioned above were maintained, at 23 to 25°C with a relative humidity of 45 to 65% under a lighting regi-

Table 1. Comparison of cecal flora of rats under CV and SPF conditions

Bacterial groups	CV Wistar (N=3)	SPF Wistar (N=3)
Bacteroidaceae	10.1 ± 0.10 (3)	10.0 ± 0.25 (3)
Fusiform bacteria	8.8 ± 0.20 (3)	
Eubacteria	9.6 ± 0.06 (3)	
Clostridia	8.0 ± 0.00 (3)	8.5 ± 0.57 (3)
Curved rods	9.1 ± 0.17 (3)	
Peptococcaceae	9.5 ± 0.45 (3)	
Bifidobacteria	9.2 ± 0.35 (3)	
Veillonellaceae	5.7 ± 0.26 (3)	7.8 ± 1.88 (3)
Lactobacilli	9.0 ± 0.30 (3)	9.7 ± 0.32 (3)
Enterobacteriaceae	6.4 ± 0.15 (3)	6.7 ± 1.28 (3)
Streptococci	6.2 ± 0.74 (3)	9.0 ± 0.30 (3)
Staphylococci	4.3 ± 0.89 (3)	5.9 ± 0.35 (3)
<i>S. aureus</i>	4.0 ± 0.62 (3)	5.0 ± 1.00 (3)
<i>Pseudomonas aeruginosa</i>		4.9 ± 1.10 (3)
Corynebacteria		
Bacilli		7.9 (1)
Total counts	10.4 ± 0.10	10.2 ± 0.15

Mean ± S.D. of log. counts of bacteria/g feces when present; figures in parentheses refer to the number of animals that harbored the organisms.

men of a 12-hr light/dark cycle, and complete air exchange 12 to 15 times/hr. They were housed in aluminum cages (breeding : 225 × 375 × 210 mm, maintenance: 315 × 470 × 200 mm), and fed commercial diet (MR-A1 and MR-A2) sterilized at 121°C for 20 min. Acidified water (pH: 2.5–3.0 adjusted with hydrochloric acid) was given *ad libitum*. SPF rats were produced by a program of random breeding.

Bacteriological culture techniques: Bacteriological procedures were essentially the same as those described by Yanabe *et al.* [23]. After serial 10-fold dilution of homogenized cecal contents under O₂-free CO₂ gas flow, the diluted samples were spread on the surface of 11 selective and four nonselective media. Egg yolk agar (Eiken, Tokyo, Japan) was used for staphylococci instead of PEES agar. Bacterial numbers were expressed as log₁₀ counts of viable bacteria per gram wet weight of cecal content.

Monitoring of pathogens: To determine the microbiological status of pathogens in the SPF colonies, rats were routinely checked in the breeding room at intervals of 1 month. Infectious agents tested in this study

Table 2. Cecal flora of two groups of LF rats inoculated with B-strains and CHF (*Clostridium*)

Bacterial groups	Inoculum	
	B-strains (N=4)	CHF (N=2)
Bacteroidaceae	10.3 ± 0.08 (4)	
Fusiform bacteria		9.1 ± 0.56 (2)
Eubacteria		
Clostridia		9.6 ± 0.21 (2)
Curved rods		
Peptococcaceae		
Bifidobacteria		
Veillonellaceae		
Lactobacilli		
Enterobacteriaceae	9.2 ± 0.36 (4)	8.7 ± 0.42 (2)
Streptococci		
Staphylococci		
<i>S. aureus</i>		
<i>Pseudomonas aeruginosa</i>		
Corynebacteria		
Bacilli		
Total counts	10.3 ± 0.08	9.7 ± 0.28

Mean ± S.D. of log. counts of bacteria/g feces when present; figures in parentheses refer to the number of animals that harbored the organisms.

were *Salmonella* spp., *Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Pasteurella pneumotropica*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, Tyzzer's organism, *Mycoplasma* spp., Sendai virus, Hantavirus, Sialodacryoadenitis virus, *Syphacia* spp., *Giardia* spp. and *Spironucleus* spp.

Results

Cecal flora of SPF and CV rats: The compositions of cecal flora of existing SPF Wistar rats and CV Wistar rats used as flora sources for GB rats are presented in Table 1. The cecal flora of CV rats was composed of Bacteroidaceae as the predominant population and various types of anaerobic bacteria as the dominant population. However, in existing SPF Wistar rats the composition of anaerobic bacterial groups was simple compared with that of CV rats, and *P. aeruginosa* was detected at high levels.

Cecal flora of LF rats and GB rats: Cecal flora of two groups of limited-flora rats inoculated with B-strains and CHF (*Clostridium*) are presented in Table 2. The flora

Table 3. Changes of cecal flora of gnotobiotic Wistar rats maintained in an isolator after administration of limited flora

Bacterial groups	Period after administration				
	2 weeks ^{a)} (N=2)	3 months ^{a)} (N=4)	8 months ^{b)} (N=3)	30 months ^{b)} (N=3)	42 months ^{b)} (N=2)
Bacteroidaceae	10.2 ± 0.00 (2)	10.0 ± 0.15 (4)	10.3 ± 0.00 (3)	10.1 ± 0.17 (3)	10.4 ± 0.20 (2)
Fusiform bacteria	8.9 ± 0.92 (2)	8.1 ± 0.42 (4)	9.0 ± 0.30 (3)	9.1 ± 0.17 (3)	9.1 ± 0.40 (2)
Eubacteria					
Clostridia	9.7 ± 0.49 (2)	8.5 ± 0.56 (4)	9.1 ± 0.40 (3)	9.5 ± 0.31 (3)	9.9 ± 0.50 (2)
Curved rods		8.2 ± 0.17 (3)	8.7 (1)	8.7 ± 0.00 (2)	
Peptococcaceae					
Bifidobacteria					
Veillonellaceae					
Lactobacilli	10.0 ± 0.14 (2)	9.2 ± 0.28 (4)	9.9 ± 0.06 (3)	9.5 ± 0.61 (3)	9.7 ± 0.15 (2)
Enterobacteriaceae	6.6 ± 1.48 (2)	5.5 ± 0.37 (4)	7.4 ± 0.12 (3)	5.5 ± 1.10 (3)	6.4 ± 1.10 (5)
Streptococci					
Staphylococci					
<i>S.aureus</i>					
<i>Pseudomonas aeruginosa</i>					
Corynebacteria					
Bacilli					
Total counts	10.6 ± 0.07	10.1 ± 0.13	10.5 ± 0.00	10.3 ± 0.26	10.6 ± 0.15

Mean ± S.D. of log. counts of bacteria/g feces when present; figures in parentheses refer to the number of animals that harbored the organisms. ^{a)}First generation. ^{b)}About 10-week-old rats were tested.

of these two groups were stabilized one month after inoculation. The results of monitoring of cecal flora of the GB rats maintained in an isolator after administration with limited flora and L-strains are shown in Table 3. In the GB rats, composition of cecal flora was very stable for a long time. Bacteroidaceae as the predominant population, clostridia, fusiform-shaped bacteria, curved rods and lactobacilli as the dominant population, and Enterobacteriaceae as the low population were detected constantly.

Colonization resistance of GB rats to *P. aeruginosa* and cecal size of GB rats: In GB rats administered B-strains, CHF and L-strains, *P. aeruginosa* was eliminated from the intestines. Two and 3 weeks after *P. aeruginosa* inoculation, *P. aeruginosa* was not detected even after enrichment in NAC broth. The cecal size of GB rats two weeks after administration of LF flora was 2.5–2.8%, and three months later was the level of CV rats (1.4–2.0%). These values were very low compared with those of LF rats with B-strains (6.8–8.1%).

Monitoring of cecal flora of SPF rats: The changes of cecal flora of SPF rats are shown in Table 4. After transfer from the isolator to a barrier-type room, strep-

tococci, staphylococci and Veillonellaceae, which had not been inoculated in GB rats, were detected, but the basal components of the intestinal flora did not change. After 13 months, streptococci were the dominant bacteria. *P. aeruginosa* and *S. aureus*, which appear to be difficult to control in a barrier environment, were not detected in the cecum of the SPF rats.

Monitoring of pathogens: Pathogens were not detected in the SPF rats during the study period.

Discussion

The present intention of the study was to establish SPF rat breeding colonies using gnotobiotic rats exhibiting colonization resistance to *P. aeruginosa* and normal physiological functions for the artificial supply of indigenous flora. Production of SPF mice using gnotobiotic techniques has been established [5, 7–9, 11,12]. Clostridia obtained from CV mouse feces are able to reduce the cecal size of gnotobiotic mice to the normal level but a cocktail of many strains of clostridia is required [8], and clostridia and lactobacilli are necessary to reduce *E. coli* populations in the intestine to normal levels [2, 5]. Combinations of Bacteroidaceae, clostridia and lactobacilli were required to eliminate *P.*

Table 4. Changes of cecal flora of SPF Wistar rats after transfer from an isolator to a barrier room

Bacterial groups	Months after transferred				
	2 months ^{a)} (N=5)	9 months ^{b)} (N=5)	13 months ^{b)} (N=5)	27 months ^{b)} (N=3)	36 months ^{b)} (N=5)
Bacteroidaceae	10.4 ± 0.08 (5)	10.3 ± 0.26 (5)	10.2 ± 0.05 (5)	10.3 ± 0.05 (3)	10.2 ± 0.07 (5)
Fusiform bacteria	9.4 ± 0.19 (5)	9.5 ± 0.35 (5)	9.3 ± 0.39 (5)	9.2 ± 0.16 (3)	9.1 ± 0.18 (5)
Eubacteria					
Clostridia	9.6 ± 0.19 (5)	9.7 ± 0.34 (5)	9.9 ± 0.18 (5)	9.7 ± 0.14 (3)	9.4 ± 0.22 (5)
Curved rods	8.7 ± 0.64 (3)	8.5 ± 0.30 (5)	8.8 ± 0.25 (4)		8.3 ± 0.41 (5)
Peptococcaceae					
Bifidobacteria					
Veillonellaceae		5.7 ± 0.30 (5)	6.9 ± 1.27 (5)	5.9 ± 0.85 (3)	4.0 ± 0.86 (5)
Lactobacilli	8.8 ± 0.25 (5)	8.1 ± 0.44 (5)	9.5 ± 0.44 (5)	9.7 ± 0.12 (3)	9.4 ± 0.21 (5)
Enterobacteriaceae	6.2 ± 0.74 (5)	4.9 ± 0.85 (5)	5.4 ± 0.66 (5)	4.8 ± 0.52 (3)	4.4 ± 0.93 (5)
Streptococci	6.8 ± 0.65 (5)	7.7 ± 0.56 (5)	9.3 ± 0.23 (5)	8.9 ± 0.28 (3)	8.2 ± 0.58 (5)
Staphylococci	4.4 ± 0.67 (5)	5.4 ± 0.72 (5)	5.4 ± 0.72 (5)	5.6 ± 0.25 (3)	4.9 ± 1.13 (5)
<i>S.aureus</i>					
<i>Pseudomonas aeruginosa</i>					
Corynebacteria					
Bacilli					
Total counts	10.5 ± 0.08	10.5 ± 0.24	10.6 ± 0.05	10.5 ± 0.00	10.3 ± 0.05

Mean ± S.D. of log. counts of bacteria/g feces when present; figures in parentheses refer to the number of animals that harbored the organisms. ^{a)} First generation. ^{b)} About 10-week-old rats were tested.

aeruginosa from gnotobiotic mice [9]. The composition of intestinal flora of CV rats is similar to that of CV mice [17, 21, 22]. In mice and rats, clostridia are reported [8, 14, 15] as the most important bacteria for normalization of gnotobiotic animals and as a colonization resistant factor [9, 20]. Lactobacilli are also considered to be an important member of the intestinal flora of both rats [22] and mice [5, 12]. However, in CV mice, *Lactobacillus acidophilus* I, *L. murinus* I a, and *L. reuteri* IVb were the most frequent organisms in the feces irrespective of the breeding colony, mouse strain and age [7]. The same species but different biovars of *Lactobacillus* were predominant in CV rats.

In the existing SPF Wistar rats, *P. aeruginosa* was detected and the composition of the cecal flora was simple compared with that of CV rats. This may be due to restrictions on contact with many intestinal bacteria because of the microbiological barrier system. The results indicated that problems might be caused by poor intestinal flora. However, the SPF rats originally administered *E. coli*, 28 strains of Bacteroidaceae, three strains of *Lactobacillus* and clostridia, showed colonization resistance to *P. aeruginosa*, in spite of a similar composition of cecal bacteria to that of existing SPF rats. These results indicate that this gnotobiotic rat

carried the minimum indispensable components of intestinal bacteria. The combination of floral bacteria harbored in the GB rats was the same as that in gnotobiotic mice with normal physiological functions [9]. With these results, we have confirmed that the gnotobiotic techniques used for gnotobiotic mice are useful for the production of gnotobiotic rats.

In the SPF rats, staphylococci, streptococci and Veillonellaceae which were not detected in the GB rats were detected at low population levels. These organisms may originate from the existing SPF rats. However, *P. aeruginosa*, *S. aureus* and bacilli which colonized the existing SPF rats were not detected. The composition of intestinal bacteria of GB rats played an important role in the elimination of *P. aeruginosa* and *S. aureus*, and were very stable for more than 3 years. The basic composition of cecal flora, mainly composed of Bacteroidaceae, clostridia, fusiform-shaped bacteria and lactobacilli, did not change for a long period, more than 3 years, and their flora become similar to those found in CV rats. Moreover, *E. coli* in the cecum were controlled at a low population level. Koopman *et al.* [13] found that the concentration of *E. coli* in the feces is the most suitable parameter for monitoring the stability of floral composition and normalization of GF

mice. Pathogens tested were not detected during this study. These results were satisfactory for an SPF rat breeding colony.

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