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Establishment of Specific Pathogen-Free Rabbit Colonies with Limited-Flora Rabbits Associated with Conventional Rabbit Flora, and Monitoring of Their Cecal Flora

Manabu YANABE[1], Masaharu SHIBUYA[1], Toshihiko GONDA[1], Hidekazu ASAI[1], Toshio TANAKA[1], Teruo NARITA[2], Katsuko SUDO[3], and Kikuji ITOH[4]

[1]Japan SLC, Inc., Hamamatsu, Shizuoka 431-1103, [2]Sankyo Co., Ltd., Shinagawa-ku, Tokyo 140-005, [3]Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108-0071, [4]Department of Veterinary Public Health, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

Abstract: *In the present study we attempted to establish specific-pathogen-free (SPF) rabbit breeding colonies with two groups of limited-flora (LF) rabbits, both ex-germfree rabbits, and their offspring. Two groups of LF rabbits associated with cecal flora of conventional (CV) rabbits produced in a previous study [Exp. Animals, submitted], were transferred to individual barrier rooms and some of the LF rabbits were accomodated in isolators to maintain the basic flora for SPF rabbits. The composition of the cecal flora of LF rabbits was stable for a long period; bacteroides remained predominant and clostridia dominant. From the SPF rabbits, different types of bacteria, e.g., enterobacteriaceae and streptococci, which could not be isolated in the isolator were detected at a low population level at an early stage in the establishment of the SPF colonies, but the basic composition of the cecal flora was mainly bacteroidaceae and clostridia and did not change over a long period, and the floral composition became similar to that of CV rabbits. The fertility and weaning rates of the SPF rabbits were satisfactory for a SPF rabbit colony. In addition, these SPF colonies were free of more than one year rabbit-specific pathogens.*

Key words: *cecal flora, ex-germfree rabbit, limited-flora rabbit, normalization, SPF rabbit*

Introduction

Specific-pathogen-free (SPF) animals are produced by using hysterectomy-derived animals and maintained in barrier type animal houses, but the barrier system eliminates not only infectious agents but also floral organisms. Consequently, because SPF animals lack the indigenous flora which resist pathogenic species or opportunistic pathogens, the artificial supply of indigenous flora is essential for hysterectomy-derived animals.

Microbiological standardization of the intestinal flora of SPF laboratory animals is required in now several research fields, but only a few reports on the production of SPF rabbits [1, 7] and on monitoring data on the intestinal flora of SPF rabbits produced from ex-GF rabbits or gnotobiotes have been published. Baba *et al.*

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Address corresponding: K. Itoh, Department of Veterinary Public Health Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

Table 1. Transfer of LF rabbits from isolators to barrier-sustained rooms

Isolator					Barrier room	
LF rabbits	Sex	Age (months)	No of animals		No. transferred	(floor area)
Expt. 3–1	Male	14*	2	→	1	
		2.5–3.0**	2	→	2	B
	Female	14*	6	→	4	(66.41 m^2)
		2.5–3.0**	5	→	5	
Expt. 3–4	Male	14*	2	→	1	
		2.5–3.0**	6	→	4	A
	Female	14*	5	→	3	(27.60 m^2)
		2.5–3.0**	4	→	4	

Migration was performed after 13 months of colonizing the cecal flora. *Ex-GF rabbits. **Offspring of ex-GF rabbits.

[1] reported that almost all the rabbits died of diarrhea after transferring hysterectomy-derived animals directly or with some intestinal bacteria into a barrier room. In a previous study, we reported the production of nine groups of limited-flora (LF) New Zealand White (NZW) rabbits associated with various components of cecal flora of conventional (CV) rabbits, and finally selected the two groups based on the survival rate and health condition of ex-GF rabbits for the establishment of SPF rabbit colonies [7]. In the present study we attempted to establish breeding colonies of SPF rabbits, which harbor normal intestinal flora, by using the two groups of LF rabbits and we monitored the cecal flora and pathogenic microbes in the SPF rabbits and checked the weaning rate to confirm the normal state as a rabbit breeding colony.

Materials and Methods

Maintenance of limited-flora (LF) rabbits: We used two groups of LF rabbits reported previously (Yanabe *et al.* submitted to Experimental Animals). Both groups were first administered six strains of Bacteroides isolated from the cecal contents of 2 to 3-week-old CV rabbits. Thereafter, the Expt. 3–1 group was inoculated with a suspension of anaerobic growth on EG agar plates injected with cecal contents of CV rabbits. The Expt. 3–4 group was inoculated with EG plate cultures of cecal contents of ex-GF mice which had been inoculated with the cecal suspension of CV rabbits. The two groups of LF rabbits were kept in large flexible vinyl isolators (1850 × 1800 × 800 mm) in the same way as GF animals and fed by restricted feeding of commercial diet (Nihon Nosan, Yokohama, Japan) sterilized by γ-irradiation at 50 kGy. Autoclaved water was given *ad libitum*. Breeding was at first started with 2 males and 7 females in the Expt. 3–1 group, and 2 males and 5 females in the 3–4 group at 8 months of age.

Transfer of ex-GF rabbits to barrier rooms: The Expt. 3–1 and 3–4 groups of LF rabbits were transferred from the isolators to barrier rooms A and B, respectively, 13 months after associated with cecal flora. Some of the LF rabbits were kept in the isolators to maintain their flora, and were bred. Table 1 summarizes the number of animals and floor space of the barrier rooms.

Maintenance of SPF rabbits: The animals were kept in the barrier unit at 25–27°C with a relative humidity of 45–65% under a lighting regimen of a 12 hr light/dark cycle, and total air exchanged 12–15 times/hr. They were housed in aluminum cages (breeding: 1230 × 625 × 450 mm, maintenance: 600 × 600 × 430 mm), and fed by restricted feeding with commercial diet (breeding: 5L68, maintaining: 5L69, PMI Feeds, Inc., USA) sterilized at 121°C for 20 min. Acidified water (pH 2.5–3.0 adjusted with hydrochloric acid) was given *ad libitum*. Breeding was performed by a rotation pairing system using 7-month-old males and 6-month-old females.

Bacterial culture techniques: Bacteriological procedures were essentially the same as those described by Mitsuoka *et al.* [5, 6] and Itoh and Mitsuoka [4], except that an anaerobic chamber was employed instead of the "plate-in-bottle" method. The diluted samples were spread on the surface of 11 selective and four nonselec-

Table 2. Methods of monitoring pathogenic microbes in SPF rabbit colonies

Pathogen	Methods
Salmonella spp.	Culture (DHL agar) of feces and Serology (agglutination test)
Pasteurella multocida	Culture (5% horse blood agar) of nasal cavity swab
Bordetella bronchiseptica	Culture (DHL agar) of nasal cavity swab and Serology (agglutination test)
Pseudomonas aeruginosa	Culture (NAC agar) of feces
Tyzzer's organism	Serology (complement fixation test)
Mycoplasma spp.	Culture (Chanock's PPLO agar) of nasal cavity swab
Sendai virus	Serology (complement fixation test)
Eimeria spp.	Concentration-flotation method using feces
Psoroptes cuniculi	Unaided eye or 20 × magnification test of crusty exudate inside ear

tive media. Egg yolk agar (Eiken, Tokyo) was used for staphylococci instead of PEES agar. Media and culture conditions are the same as in the previous report [7]. The numbers of bacteria were expressed as $\log_{10}$ counts of viable bacteria per gram wet weight of cecal content.

Monitoring of pathogens: To determine the microbiological status of the SPF colonies, the rabbits were examined in the two breeding rooms at intervals of 3 months. Test samples were taken from the living rabbits, i.e. ear vein blood, fresh feces, mucus membrane from nasal cavity swabs and crusty exudate from the inner ear. These samples were tested for infections with *Salmonella* spp., *Pasteurella multocida*, *Bordetella bronchiseptica*, *Pseudomonas aeruginosa*, Tyzzer's organism, *Mycoplasma* spp., Sendai virus, *Eimeria* spp. and *Psoroptes cuniculi*. Checking methods are summarized in Table 2.

Fertility: To confirm the fertility of the SPF rabbits as a breeding colony, the pregnancy rate, birth rate, litter size and weaning rate were examined.

Statistical analysis: Significance of differences between mean values was tested with Student' *t*-test.

Results

Monitoring of cecal flora in LF rabbits: The results of the monitoring of cecal flora in the two groups of LF rabbits reared and bred in isolators are shown in Tables 3 and 4. In the Expt. 3–4 group, the composition of cecal flora was very stable for 69 months. Bacteroidaceae as the predominant population, clostridia as the dominant and bacilli as the low population were constantly detected. Detection of eubacteria varied in this group, but in the Expt. 3–1 group, staphylococci and corynebacteria were also detected. Thirty months after the Expt. 3–1 group was produced, enterobacteriaceae was also detected. We therefore concluded that this isolate had been contaminated and discontinued this group.

Monitoring of cecal flora in SPF rabbits: The changes in cecal flora in SPF rabbits are shown in Tables 5 and 6. In both SPF rabbits, changes in floral composition were similar except for corynebacteria. At six months after LF rabbits were transferred from each isolator to barrier-type rooms, enterobacteriaceae, streptococci and staphylococci, which had not colonized in the intestines of LF rabbits, were detected in both colonies. Thereafter, staphylococci disappeared after 18 months. Eubacteria and peptococcaceae were detected until 18 months and not detected thereafter. After 21 months, the composition of cecal flora was stable in both colonies and became similar to that of CV rabbits.

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

Fertility: There were no statistical differences in fertility among colonies A and B and the original CV NZW colony (Table 7).

Discussion

The present study was aimed at the establishment of SPF rabbit breeding colonies by using LF rabbits for the artificial supply of indigenous flora, and the monitoring of their cecal flora, pathogenic microbes and health condition to evaluate LF rabbits as a source of intestinal flora in SPF rabbits.

In a previous study, we produced two groups of ex-GF NZW rabbits associated with cecal flora of CV rabbits as a source of intestinal flora for SPF colonies, and maintained them in isolaters [7]. Unfortunately,

Table 3. Cecal flora in LF rabbits maintained in an isolator (Expt. 3–4) after administration of cecal bacteria† from conventional rabbits

Bacterial group	Period after administration					
	6 months (N=2)	12 months (N=4)	30 months (N=2)	33 months (N=4)	47 months (N=4)	69 months (N=4)
Bacteroidaceae	9.9 ± 0.2 (2)*	9.7 ± 0.2 (4)	10.1 ± 0.1 (2)	10.2 ± 0.1 (4)	10.1 ± 0.3 (4)	10.4 ± 0.1 (4)
Eubacteria		7.9 ± 0.4 (3)	8.0 ± 0 (2)			
Clostridia	9.5 ± 0.1 (2)	9.1 ± 0.5 (4)	9.8 ± 0.1 (2)	9.7 ± 0.1 (4)	8.8 ± 0.9 (4)	9.8 ± 0.5 (4)
Spirillaceae						
Peptococcaceae						
Bifidobacteria						
Lactobacilli						
Enterobacteriaceae						
Streptococci						
Staphylococci						
Pseudomonas aeruginosa						
Corynebacteria						
Bacilli	3.7 ± 1.5 (2)	3.8 ± 1.1 (4)	5.5 ± 0.3 (2)	5.9 ± 0.1 (4)	5.9 ± 1.2 (4)	5.5 ± 0.2 (4)
Yeasts						
Total counts	10.0 ± 0.3	9.8 ± 0.2	10.3 ± 0.1	10.3 ± 0.1	10.1 ± 0.3	10.5 ± 0.2

†EG plate cultures of cecal contents of ex-GF mice which had been inoculated with cecal suspension from CV rabbits. *Means ± S.D. of log. counts of bacteria/g feces when present; figures in parentheses are the numbers of animals that harbor the organisms.

Table 4. Cecal flora of LF rabbits maintained in an isolator (Expt. 3–1) after administration of cecal bacteria† from conventional rabbits

Bacterial group	Period after administration		
	6 months (N=2)	12 months (N=4)	30 months (N=2)
Bacteroidaceae	9.8 ± 0 (2)*	10.1 ± 0.5 (4)	10.1 ± 0.1 (2)
Eubacteria	8.6 (1)	8.8 ± 0.6 (2)	
Clostridia	8.3 (1)	8.5 ± 0.9 (4)	9.4 ± 0.1 (2)
Spirillaceae			
Peptococcaceae			
Bifidobacteria			
Lactobacilli			
Enterobacteriaceae			7.4 ± 0.5 (2)
Streptococci			
Staphylococci	5.9 ± 0.6 (2)		5.8 ± 0 (2)
Pseudomonas aeruginosa			
Corynebacteria	6.7 ± 0.6 (2)	5.4 ± 1.7 (4)	7.8 ± 0.4 (2)
Bacilli	5.9 ± 0.6 (2)	3.8 ± 1.0 (4)	
Yeasts			
Total counts	9.9 ± 0.1	10.1 ± 0.5	10.2 ± 0

†EG plate cultures which were injected with cecal contents of CV rabbits. *Mean ± S.D. of log. counts of bacteria/g feces when present; figures in parentheses are the numbers of animals that harbor the organisms.

we had to discontinue one of them because of contamination. In another group (Expt. 3–4) composition of the cecal flora was stable for a long time in the isolator. These results indicate that ex-GF rabbits and their offspring kept in an isolator can provide indigenous flora for SPF rabbit colonies, and also suggest that the intestinal flora harbored in the intestines of these animals may be of the correct composition to maintain the nor-

Table 5. Changes in cecal flora in SPF rabbits in breeding colony A derived from LF rabbits (Expt. 3–4)

Bacterial group	Just transferred*	Months after transfer				
	(N=4)	6 months (N=3)	18 months (N=5)	21 months (N=5)	36 months (N=5)	54 months (N=3)
Bacteroidaceae	9.7 ± 0.2 (4)**	10.0 ± 0.2 (3)	9.9 ± 0.2 (5)	10.2 ± 0.2 (5)	10.5 ± 0.3 (5)	10.7 ± 0.2 (3)
Eubacteria	7.9 ± 0.4 (3)	8.8 ± 0.2 (2)	8.0 (1)			
Clostridia	9.1 ± 0.5 (4)	9.2 ± 0.2 (3)	9.5 ± 0.2 (5)	9.6 ± 0.1 (5)	9.7 ± 0.2 (5)	10.0 ± 0.5 (3)
Spirillaceae						
Peptococcaceae		9.3 ± 0.1 (2)	8.7 ± 4.5 (5)			
Bifidobacteria						
Lactobacilli						
Enterobacteriaceae		4.3 ± 1.5 (3)	3.6 ± 0 (2)	3.6 ± 0.6 (3)	3.8 ± 0.3 (4)	3.9 ± 0.5 (3)
Streptococci		4.8 ± 1.5 (2)		4.0 ± 1.2 (4)	3.9 ± 0.3 (5)	4.4 ± 0.8 (3)
Staphylococci		3.8 ± 1.4 (3)	3.0 ± 0 (2)			
Pseudomonas aeruginosa						
Corynebacteria						
Bacilli	3.8 ± 1.1 (4)	3.8 (1)	3.5 (1)	3.6 ± 0.4 (3)		4.4 ± 0.9 (3)
Yeasts						
Total counts	9.8 ± 0.2	10.1 ± 0.2	10.1 ± 0.1	10.3 ± 0.1	10.6 ± 0.2	10.8 ± 0.3

*See 12 months in Table 4. **Mean ± S.D. of log. counts of bacteria/g feces when present; figures in parentheses are the numbers of animals that harbor the organisms.

Table 6. Changes in cecal flora in SPF rabbits in breeding colony B derived from LF rabbits (Expt. 3–1)

Bacterial group	Just transferred*	Months after transfer				
	(N=4)	6 months (N=3)	18 months (N=5)	21 months (N=5)	36 months (N=5)	54 months (N=3)
Bacteroidaceae	10.1 ± 0.5 (4)**	9.9 ± 0.2 (3)	9.7 ± 0.3 (5)	10.1 ± 0.2 (5)	10.7 ± 0.1 (5)	10.8 ± 0.2 (3)
Eubacteria	8.8 ± 0.6 (2)	7.8 ± 0.7 (2)				
Clostridia	8.5 ± 0.9 (4)	8.5 ± 0.2 (2)	9.2 ± 0.2 (5)	9.3 ± 0.3 (5)	9.6 ± 0.4 (5)	10.1 ± 0.2 (3)
Spirillaceae						
Peptococcaceae			8.3 ± 1.1 (2)			
Bifidobacteria						
Lactobacilli						
Enterobacteriaceae		4.5 ± 0.6 (3)	3.3 ± 0.4 (5)	5.5 ± 0.6 (3)	3.4 ± 0.3 (4)	4.2 ± 0.1 (3)
Streptococci		7.7 ± 0.3 (3)	3.5 ± 0.4 (5)	5.5 ± 0.6 (4)	4.0 ± 0.6 (5)	4.0 ± 0.5 (3)
Staphylococci		4.2 ± 1.2 (3)				
Pseudomonas aeruginosa						
Corynebacteria	5.4 ± 1.7 (4)		4.0 ± 1.1 (4)	5.5 ± 0.8 (2)		
Bacilli	3.8 ± 1.0 (4)		3.3 (1)			4.1 ± 1.0 (3)
Yeasts						
Total counts	10.1 ± 0.5	9.9 ± 0.2	9.8 ± 0.3	10.2 ± 0.2	10.8 ± 0.1	10.9 ± 0.1

*See 12 months in Table 4. **Mean ± S.D. of log. counts of bacteria/g feces when present; figures in parentheses are the numbers of animals that harbor the organisms.

Table 7. Fertility of SPF rabbits derived from LF rabbits

Breeding colony	No. of pairs	Pregnancy rate	Birth rate	Litter size mean ± S.D.	Weaned mean ± S.D.	Weaning rate
A	71	84%	100%	5.2 ± 2.1	4.5 ± 2.4	85.9%
B	80	77%	100%	4.8 ± 1.8	3.7 ± 2.3	80.4%
Cont.*	252	70%	92%	No data**	4.4**	79.6%

*Original CV NZW rabbits. **No data for individuals.

mal physical state.

In SPF rabbits, anaerobic and aerobic bacteria of environmental origin were detected at an early stage of the SPF colonies, but the basic composition of cecal flora, mainly bacteroidaceae and clostridia, had not changed, and their flora finally became similar in composition to that of CV rabbits [7]. Eubacteria, peptococcaceae, staphylococci and corynebacteria were finally eliminated, and enterobacteriaceae, streptococci and bacilli were kept at low population levels. These results indicated that bacteroidaceae and clostridia are essential floral organisms in rabbits, and the other organisms eliminated from or suppressed in the intestine do not appear to be essential in SPF rabbits. Rabbits readily suffer from diarrhea, e.g. when they are transported or their diet is changed. Under such stressful conditions, aerobic bacteria, especially enterobacteriaceae, abnormally increase in the intestines (unpublished data). The mortality of GF and ex-GF rabbits, which were inoculated with CV rabbit feces and combinations of *Bacteroides* sp., *Eubacterium* sp. and *Streptococcus* sp., was 47–100% after they were transferred into barrier rooms, and the numbers of aerobes, especially enterobacteriaceae, were very high [1]. It is very important to evaluate the LF rabbit as a source of intestinal flora for SPF rabbits that the numbers of aerobes were suppressed to a low level in the intestines of these SPF rabbits. Coryne-bacteria detected from ex-GF (Expt. 3–1) and SPF (B colony) did not agglutinate the antiserum of the pathogenic strain of *Corynebacterium kutscheri.*

The fertility of SPF rabbits did not differ from that of the original CV NZW rabbits, and no pathogens were present among the organisms tested in this study, although CV rabbits used as donors of cecal flora had *Bordetella bronchiseptica.* These results were satisfactory for an SPF rabbit breeding colony.

In the present study we confirmed that colonization of combinations of indigenous anaerobes in the intestine are indispensable for SPF rabbits, but these colonies may be necessary to prevent inbreeding depression of the rabbits. As a countermeasure, new ex-GF rabbits, which are hysterectomy-derived and have intestinal flora transferred from stocked LF rabbits by cohabitation, should be added periodically to the SPF colony.

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