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Rearing and breeding of germ-free mice for over 1 year in a sealed positive pressure cage system

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Abstract

Germ-free mice and gnotobiotic mice have long been used as models in various research areas. Historically, they were reared and used for studies of flexible film isolators, but such isolators have several operational disadvantages. To overcome these disadvantages, a new individually ventilated cage system that promises more standardized cage conditions and reduced operational costs for maintaining germ-free or gnotobiotic mice has been developed. However, in the context of this system, only short periods (2–12 weeks) of rearing and experimental use of germ-free or gnotobiotic mice have been reported. In this report, we describe the rearing and breeding of 117 germ-free mice for over 1 year using the Sentry SPP cage, a sealed positive pressure isolation cage dedicated to gnotobiology.

Key Words: Germ-free mouse, Rearing and breeding, Sealed positive pressure isolation cage

The first germ-free animal model, featuring guinea pigs reared under germ-free conditions, was developed to study host-microbial interactions[9]. Thereafter, techniques for the rearing and maintenance of healthy germ-free animals took approximately 50 years to establish. The first successful rearing of germ-free mice was reported in 1959[13]. Since then, gnotobiotic mice (germ-free mice inoculated with single strains or mixtures of known microorganisms) have been used as models in various areas of research, including obesity[6,17,18], metabolism[2,8], autoimmune diseases[7,19], central nervous system diseases[3,10], and bone homeostasis[15].

Germ-free and gnotobiotic mice are usually reared in flexible film isolators. However, there is a risk of contaminating all cages within the isolator if an error in isolator handling occurs. Isolators have another disadvantage when research necessitates parallel inoculation of germ-free mice with a variety of microorganisms: the isolator system mandates that each group be individually housed in a different isolator to prevent any cross-contamination between study groups, which is space-intensive and limits research throughput. To overcome these disadvantages, positive

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Fig. 1. Sentry Sealed Positive Pressure (SPP) cage system and personal protective equipment. a. Sentry SPP cage. b. SC-Plus Safety Cabinet. c. Rack for Sentry SPP cages. d. Sani-Rack. e. Intra-cage HEPA filter. f. Transfer chamber. g. The SC-Plus Safety Cabinet and the transfer chamber are sterilized by spraying them with soft acidic hypochlorite solution. h. SPP QDX device. i. The sterile operator wears a mask, hood, and sterile surgical gown. j. The sterile operator also wears long sterile gloves. k. The assistant wears a mask, hood, and sterile gloves, and assists the sterile operator. l. The assistant returns the changed cage from the cabinet to the rack.

pressure Isocages (Tecniplast SpA, Buguggiate, Varese, Italy) were used; successful maintenance of multiple groups of gnotobiotic mice in Isocages for 2 weeks was achieved[5]. Another group reported successful rearing of germ-free mice for 12 weeks using this system[12].

In the present report, we describe our experience with rearing and breeding germ-free mice for over 1 year using the Sentry Sealed Positive Pressure (SPP) isolation cage system (Allentown, Inc., Allentown, NJ, USA). This study was approved and overseen by the Animal Experiments Committee of RIKEN (Wako, Saitama, Japan), and was conducted in accordance with the Institutional Guidelines for Experiments using Animals.

Four- to eight-week-old germ-free C57BL/6NJcl mice (51 males and 16 females) were delivered in four shipments from CLEA Japan, Inc. (Meguro-ku, Tokyo, Japan). The purchased germ-free mice were transferred from shipment boxes into sterile Sentry SPP cages (Fig. 1a) in an SC-Plus Safety Cabinet (Allentown, Inc.) (Fig. 1b). Twenty-one male and five female mice were singly housed, and the other mice were group-housed (2–6 mice per cage). After 1 month of habituation, four pairs were made and breeding commenced.

The animal holding room was maintained at 21–25°C and 45–65% humidity. The air was changed 60 times per hour in the holding room. The floor of the room was cleaned with soft acidic

hypochlorite solution (Serius; OSG Corporation Co., Ltd., Kita-ku, Osaka, Japan) every weekday, and the ceiling and walls were cleaned with this solution once a week. Soft acidic hypochlorite solution is known to have efficacy for inactivating various microorganisms[11,16] and is used for daily cleaning in many animal facilities. Sentry SPP cages and a rack (Fig. 1c) were obtained from Allentown, Inc. In this system, HEPA-filtered air is supplied into the SPP cages and the exhaust air is also released into the room through a HEPA filter. The air is changed 40 times per hour in SPP cages. Using a Sani-Rack (Allentown, Inc.) (Fig. 1d), clean Sentry SPP cages were autoclaved with bedding (TEK-FRESH; ENVIGO, Madison, WI, USA), an intra-cage HEPA filter (Fig. 1e), and an empty water bottle. Reverse osmosis (RO) water was autoclaved in separate 1-L bottles and added to the sterile bottles in the cages. Rodent chow (CRF-1; Oriental East Co., Ltd., Itabashi-ku, Tokyo, Japan) irradiated with 50 kGy gamma rays was added to the sterile cages. Cage changes were always conducted in the SC-Plus Safety Cabinet. All of the sterile instruments, including the Sentry SPP cages, were introduced into the cabinet through the transfer chamber (Fig. 1f), which seamlessly integrates with the cabinet, after sterilizing the outside of the instruments with soft acidic hypochlorite solution. The SC-Plus Safety Cabinet and the transfer chamber were sterilized by spraying with soft acidic hypochlorite solution 10 min prior to use (Fig. 1g). The proper functioning of the self-closing supply, and exhaust ports on the Sentry SPP cages were checked with an SPP QDX device (Allentown, Inc.) (Fig. 1h) before returning them to the rack. Two operators were needed at all times to carry out mouse husbandry: the "sterile operator" (Fig. 1i and 1j) wore a sterile surgical gown and sterile sleeve cuffs, and the "assistant" (Fig. 1k and 1l) wore sterile gloves and carried all equipment, including SPP cages, between the rack and the transfer chamber or the cabinet. Both personnel wore facemasks and hoods. Cage changes were generally conducted once a month.

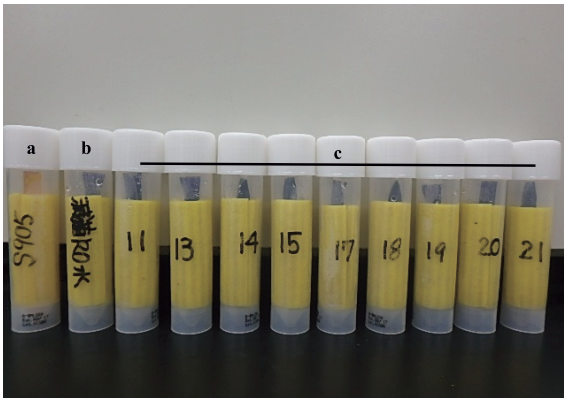

Fig. 2. SensiMedia after 7-day culture. a. Positive control (feces from SPF mice). b. Negative control (sterile reverse osmosis water). c. Fecal samples from germ-free mice.

Single-housing cages were changed every 2 months. Fresh fecal samples, soiled bedding, swabs from cages, and RO water from the drinking bottles were collected at cage changes and tested for germ-free status using aerobic and anaerobic cultures and microscopic analysis.

Microbiological tests to confirm germ-free status were conducted by bacteriology in external and internal laboratories. External bacteriology was conducted by the ICLAS Monitoring Center (Kawasaki, Kanagawa, Japan). In the external tests, cooked meat broth and thioglycollate medium were used for anaerobic bacterial culture, whereas heart infusion broth was used for aerobic culture. All bacterial cultures were incubated at 37°C or room temperature for 14 days. For fungal culture, potato dextrose broth inoculated with samples was incubated at room temperature for 14 days. Gram-staining was also conducted on fecal samples. In the internal tests, only aerobic culture using SensiMedia (A-SML024-10; MicroBio Corporation, Sendai, Miyagi, Japan) was conducted. For negative controls, sterile RO water was used, and for positive controls, feces from SPF mice were used. After 7 days of culture, the negative controls and fecal samples from germ-free mice remained blue in color, whereas the positive controls turned orange (Fig. 2). The results of the culture tests are given in Table 1. Gram-positive bacteria were

Table 1. The results of culture tests

External/ Internal	Aerobic/ Anaerobic	Culture media	Culture temperature	Sample	2017 Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	2018 Jan.	Feb.	Mar.
External test	Anaerobic	Cooked meat broth	37 °C	Fece	–*	–	–	–	0/10	–	–	–	–	–	–	–	–	0/6
				Bedding	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/6
				Swab	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/6
			Room Temperature	Fece	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/1
				Bedding	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/2
				Swab	–	–	–	–	0/10	–	–	–	–	–	–	–	–	–
		Thioglycollate medium	37 °C	Fece	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/6
				Bedding	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/6
			Room Temperature	Fece	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/2
				Bedding	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/2
	Aerobic	Heart infusion broth	37 °C	Fece	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/6
				Bedding	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/6
				Swab	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/6
			Room Temperature	Fece	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/1
				Bedding	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/6
				Swab	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/2
		Potate dextrose broth	Room Temperature	Fece	–	–	–	–	0/10	–	–	–	–	–	–	–	–	–
				Bedding	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/1
				Swab	–	–	–	–	0/10	–	–	–	–	–	–	–	–	0/2
Internal test	Aerobic	SensiMedia	Room Temperature	Fece + RO water in the bottle	0/20	0/43	0/17	0/32	0/16	0/38	0/36	0/45	0/29	0/51	0/34	0/44	0/23	–

The results are represented as number of positive sample/number of toral samples.
* not tested.

seen in some fecal smear preparations in external examination (Fig. 3). These bacteria were thought to be dead bacteria derived from irradiated chow.

As shown in Table 1, the results of all culture tests were negative. The results of the external tests in June 2017 and March 2018 indicated germ-free status of 10 and 6 arbitrarily chosen cages from among the total of 32 and 23 cages, respectively. The results of the internal tests indicated that all mice were negative for aerobes and facultative anaerobes, whereas obligate anaerobes were not evaluated in the internal tests. Because obligate anaerobes cannot exist in aerobic environments, there is little possibility that contamination with an obligate anaerobe would have occurred, suggesting that germ-free status was maintained in all cages throughout the entire period. However, the possibility that contamination with spore-forming obligate anaerobes, such as *Clostridium* spp., may have occurred cannot be ruled out.

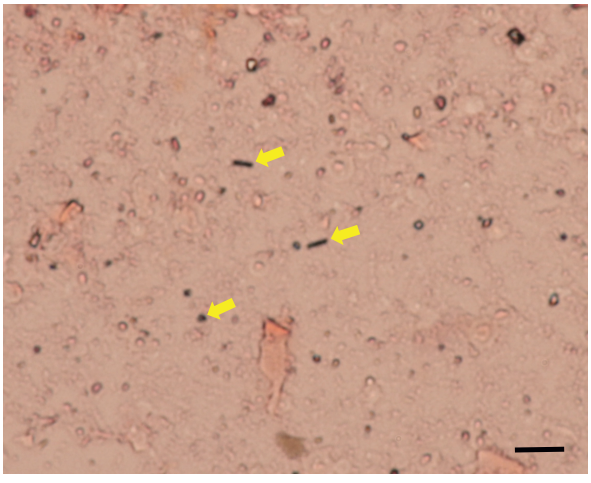

Fig. 3. Representative photomicrograph of gram-staining of a fecal smear preparation. Gram-positive bacteria are indicated with yellow arrows. Scale bar = 5 μm.

The total number of germ-free mice reared in the Sentry SPP system over the study period was 117. A total of 67 mice were purchased from a breeder, and 50 mice were born from four pairs at our facility. A total of 16 mice died during the study period; 7 mice died due to twisting of the bowel (Fig. 4a), 2 died from ruptured ceca (Fig. 4b), 2 died from malfunction of other organs due to oppression by the extremely enlarged cecum (Fig. 4c), 1 died from malnutrition due to malocclusion, and the causes of death of the other 4 mice were undetermined. Cecal enlargement in germ-free mice is caused by retention of water attracted to the accumulated mucus and undigested fibers in the cecal lumen[20]. In the present study, an approximately 6-fold enlargement of the cecum was observed in healthy germ-free mice compared to SPF mice (Fig. 4d),

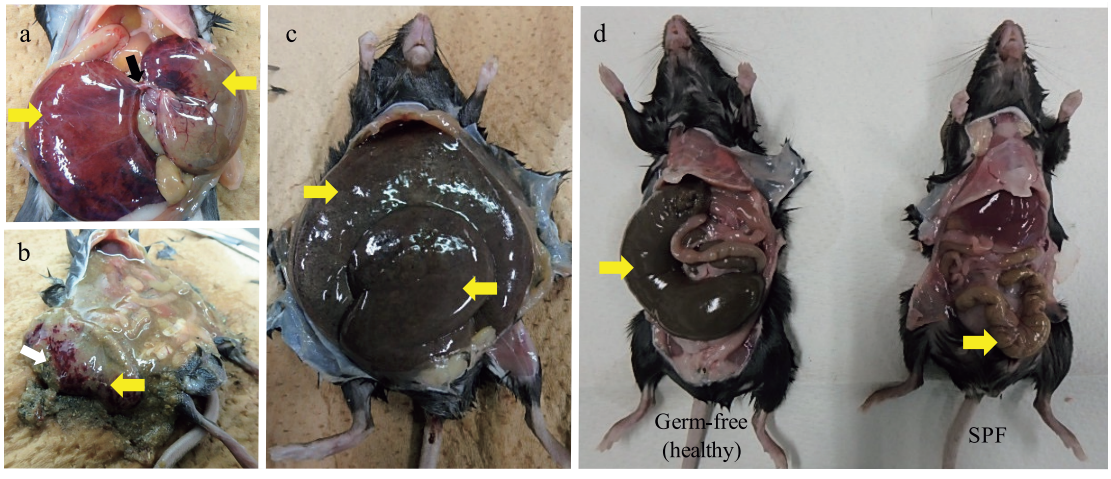

Fig. 4. Representative photographs of mouse ceca. The ceca are indicated by yellow arrows. a. Twisting of the cecum itself. The black arrow indicates the twist. b. Ruptured cecum. The white arrow indicates the cleft. c. Extreme enlargement of cecum. d. An approximately 6-fold enlargement of the cecum is observed in a healthy germ-free mouse compared to an SPF mouse.

as previously described[1]. Cecal enlargement appears to be the underlying cause of most of the deaths observed in this study. Gordon *et al.* reported that the weight of the cecum of germ-free mice at death is approximately 15 times the conventional value on average[4]. Twisting of the bowel, especially at the ileocecal junction, due to an enlarged cecum has also been reported as a frequent cause of death in germ-free mice[4,14]. The thinner wall of the enlarged cecum is a feature known of germ-free mice[1], and in this study, the thin walls of extremely large ceca presumably resulted in rupture. Malfunction of other organs, especially the lungs, due to oppression by extremely enlarged ceca was presumed as another cause of death in the present study. The ages of the two mice that died from this cause was 45 and 54 weeks old, suggesting that extreme enlargement of the cecum increases with age. Aside from the 16 mice that died, all of the mice were reared without any physical problems. In conclusion, the Sentry SPP system allowed us to rear and breed germ-free mice for 1 year without bacterial contamination.

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