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Creation of an experimental rearing environment for microbiome animal research using an individually ventilated cage system and bioBUBBLE enclosure

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Abstract: To avoid microbial contamination risk, vinyl film isolators are generally used in animal microbiome experiments involving germ-free (GF) mice and/or gnotobiotic (GB) mice. However, it can take several months to gain expertise in operating the isolator competently. Furthermore, sterilization and sterility testing, which are essential for isolator preparation, can take more than 20 days. Hence, we built an experimental rearing environment that combines an individual ventilation cage system and a bioBUBBLE clean room enclosure to easily set up an experimental animal microbiome environment for animal facilities. In this work, a three-step evaluation was conducted. First, we examined whether GF mice can be maintained in this rearing environment without bacterial contamination. Next, we examined whether GF and GB mice can be maintained without cross-contamination in one individual ventilation cage rack. Finally, we tested whether GF mice can be maintained in a biological safety cabinet controlled by negative pressure. In our series of experiments, no microbial contamination occurred over more than 3 months. These results indicated that our rearing system that combines the individual ventilation cage and bioBUBBLE systems can be used not only for experiments with GF mice but also for Biosafety Level 2 experiments that handle bacteria. Our system can mitigate various disadvantages of using vinyl film isolators. In conclusion, we established an experimental method with improved working time and efficiency compared with those of the previous vinyl isolator method.
Key words: bioBUBBLE enclosure, germ-free, gnotobiotic, individually ventilated cages, microbial animal research

Introduction

Microbiomes existing in and on humans, such as those in the intestinal tract, skin, and oral cavity, are thought to be involved in health and disease. Dysbiosis of the microbiome can cause a variety of illnesses, including digestive disorders [1, 2], diabetes [3], metabolic disorders [4], and cancer [5]. Recently, many experiments have been conducted, using human microbiota-associated (HMA) mice, with human stool administered to germ-free (GF) [6] and gnotobiotic (GB) mice inoculated with bacteria isolated from the gut [7]. In these animal experiments, the vinyl isolator (VI) was the only rearing environment that reduced microbial contamination. However, since the VI is a special breeding device, it requires thoroughly trained personnel. It takes approximately half a year to equip inexperienced caretakers with the competence to operate the VI for the maintenance of GF mice. It is difficult for even a skillful worker to carry out delicate experimental operations due

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to the need to wear thick gloves. In addition, prior to starting an experiment using GF mice, it is necessary to sterilize the isolator and conduct a sterility test, which can take more than 20 days.

In recent years, the use of individually ventilated cage (IVC) rack systems has increased in laboratory rodent facilities. These systems can prevent contamination when infected and non-infected mice are kept together in separate cages in the same IVC rack [8]. Previous studies have shown that it is possible to maintain both GF and GB mice in IVC systems for up to 12 weeks [9–11]. bioBUBBLE clean rooms are positive-pressure enclosures. They can maintain a high degree of cleanliness in a clean room by blowing a large amount of air filtered by a high-efficiency particulate air (HEPA) filter. Based on the above, we aimed to build a highly practical environment for rearing and experiments using a bioBUBBLE (bB) which, when combined with an IVC, can be used to construct a clean room.

Three evaluations were conducted with the aim of expanding the experimental applications of this system. In the first evaluation, we introduced this system into a non-barrier animal room and attempted to breed the GF mice. Next, we evaluated whether this system can be used to perform GB mouse experiments with various bacterial species. We also attempted to use rearing cages for the GF mice and rearing cages for GB mice colonized with ASF [12] at the same time in a single IVC rack. Lastly, we evaluated whether this system could be used for microbiome animal experiments conducted at Biosafety Level 2 (BSL2). In an infected animal room under negative pressure control, we created an environment in which a negative pressure controlled biosafety cabinet was enclosed in a bB to maintain the GF mice.

Materials and Methods

Ethics

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Central Institute for Experimental Animals (CIEA; Certification Number: 19045A) and were performed in accordance with the CIEA guidelines.

Mice

The GF and ASF-colonized mice used in this study were from the ICR-derived inbred strain IQI/Jic maintained in CIEA. All animals were kept in a controlled environment with a 12 h light/dark cycle (lights switched on at 7:00 a.m.). The mice were group housed (3–5 per cage) and given access to food and water *ad libitum*.

Constitution of the rearing and experimental environment using IVC and bB

A highly airtight IVC system (CLEA Japan Inc., Shizuoka, Japan) (Fig. 1) and bB (bioBUBBLE Inc., Fort Collins, CO, USA) were used in this study. The IVC rack was placed inside the bB, and the bB containing the IVC rack was installed in a conventional non-barrier animal room (Fig. 2a). As shown in Fig. 2b, the bB consisted of a dressing room and a main room attached to a clean working station (WS) (W1,430 × D860 × H1,000 mm) and a pass box. The IVC and bB were also installed in a BSL2 animal room that was controlled with a negative pressure valve. The size of bB and working station could be customized according to the breeding room. The IVC and the negative-pressure biosafety cabinet (SCV-1309EC IIA2, Hitachi Industrial Equipment Systems Co., Ltd., Tokyo, Japan) were placed in the bB (Fig. 3).

Sterilization of the breeding equipment

The containment cages, water bottle, and food were sterilized by autoclave. Their placement is shown in Fig.

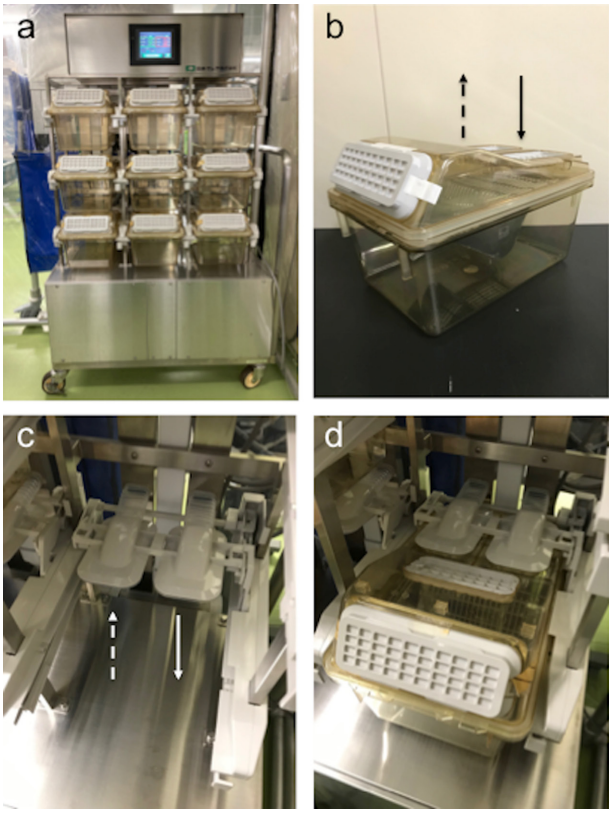

Fig. 1. The movable individual ventilation cage (IVC) system was used in this study. a) The positive and negative pressure-controlled IVC housing. b) The IVC cage. The straight arrow indicates air intake, while the dotted arrow indicates air exhaust. c) The inlet duct before setting up the cage. The arrows indicate the same information as in panel b. d) The IVC cage after being set up.

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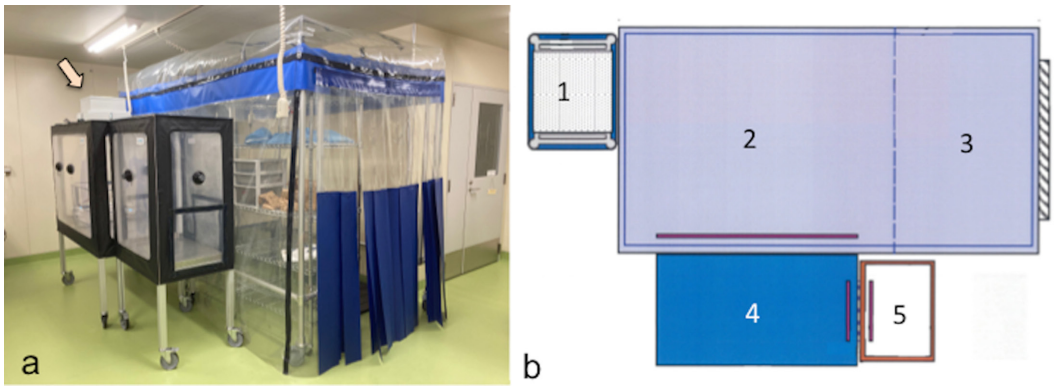

Fig. 2. The bioBUBBLE (bB) enclosure was set up in a conventional animal room. (a, b) The bB enclosure consisted of the following five parts: (1) a power unit with a high-efficiency particulate air (HEPA) filter, (2) a clean room and (3) a dressing room divided by a main room (W3,200 × D1,700 × H2,100 mm) with a strip divider (indicated by a dotted line; total size of the clean and dressing rooms: W3,200 × D1,700 × H2,100 mm), (4) a working station (W1,430 × D860 × H1,000 mm) supplied air individually (power unit indicated by an arrow in panel a), and (5) a pass box (W860 × D600 × H1,000 mm) attached to the working station with a zippered connection.

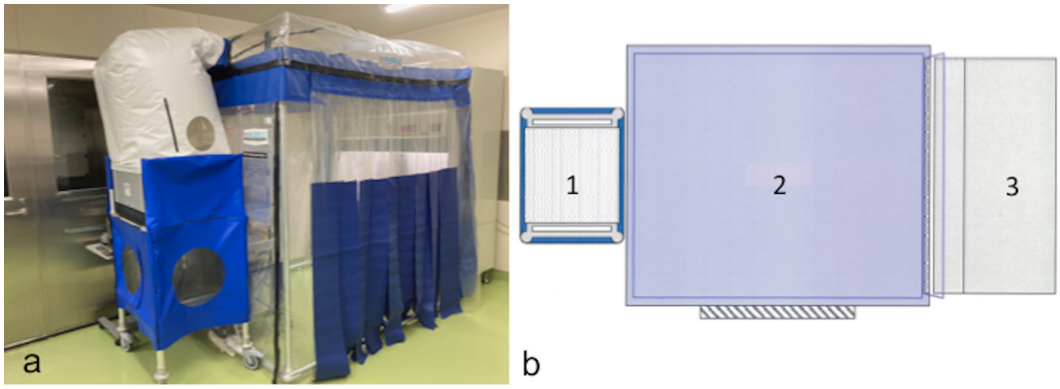

Fig. 3. The safety cabinet was set up in the bioBUBBLE (bB) in the Biosafety Level 2 animal room. (a, b) The bB enclosure consisted of the following three parts: (1) a power unit with a high-efficiency particulate air (HEPA) filter, (2) a clean room (W1,960 × D1,660 × H1,950 mm), and (3) a safety cabinet (W1,500 × D780 × H2,035 mm), which was connected to the bB with a hook and loop fastener connection.

4a. To allow heat dissipation, lids were not tightly closed (Fig. 4b). The cages were double wrapped in two sterile bags. A preliminary test was conducted under these packing conditions, and the sterilization conditions were estimated to be 127°C and 40 min according to the temperature data of a data logger (Fig. 4c). For water sterilization, a heat-resistant container filled with hot water (Fig. 4d) was also double wrapped in sterilization bags. Based on the same preliminary test as described above, 127°C and 90 min were set as the sterilization conditions (Fig. 4e). The sterilized equipment was placed in the pass box, sprayed with MB-10 (500 ppm, Quip Labs, Wilmington, DE, USA), and kept overnight in the pass box until it was used (Fig. 4f). Dust-free clothes, masks, and gloves were sterilized using the same conditions as the IVC. Workers changed their clothes in the dressing room of the bB before entering the main room wearing double gloves, wherein the first pair of gloves were taped to the wrist.

Transfer procedures for the GF/GB mice from the VI to the IVC

GF or GB mice in a VI were placed in a shipping container (W181 × D253 × H83 mm; Fuji-tokaishizai Inc., Japan), double wrapped in sterilized paper bags, and carried out through a transfer sleeve. To transfer into the bB, the outer paper bag was removed, sprayed with MB-10, and placed in the pass box. After 10 min, the inner paper bag was removed, and the shipping container was placed on the WS. Mice were moved from the container to the IVC using forceps.

Evaluation 1

The breeding environment of the IVC and the bB were

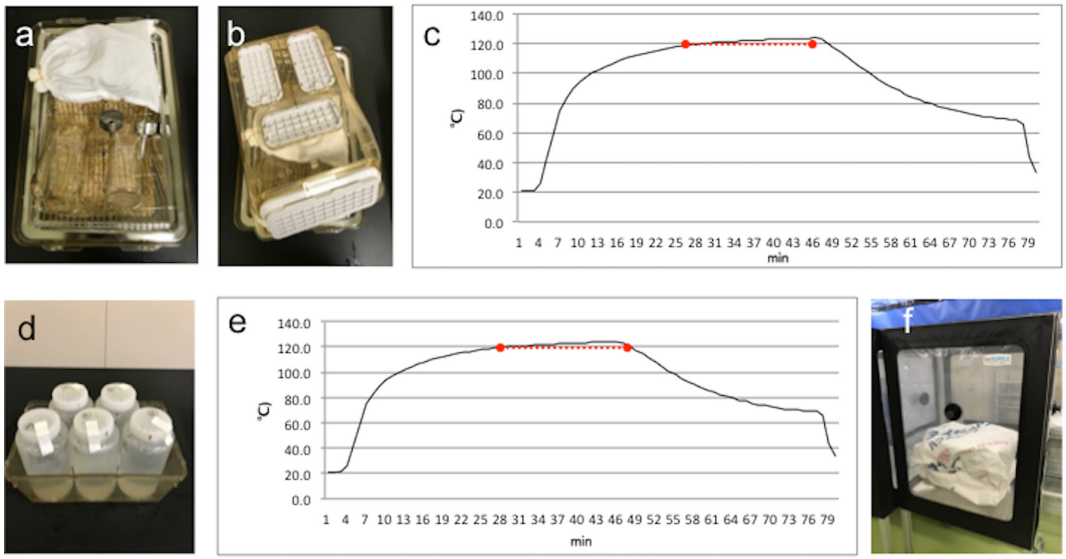

Fig. 4. Sterilization of the breeding equipment. (a) The individual ventilation cage (IVC) included bedding, empty water bottles, and the subdivided diets. (b) The IVC cage top was intentionally displaced with respect to the IVC cage, which was placed in a sterile bag. (c) The conditions of the autoclave were monitored by a thermometer installed in the cage, which confirmed sterilization. The red line indicates complete sterilization. (d) Hot water was used to fill 500 ml autoclavable plastic bottles, which were placed in a sterile bag. (e) The temperature profile in the water chart was similar to that in panel c. (f) The sterilized breeding equipment that was in the pass box is shown.

set up in a non-barrier animal room to test how long the GF mice could be maintained in a GF state. Three IVCs, which was the maximum capacity of the WS, were used in this experiment. The WS was sprayed with MB-10 before cage changes (Fig. 5a). Before the three IVCs were brought into the WS, their exteriors were sprayed with MB-10 (Fig. 5b). The WS was sprayed with MB-10 (Fig. 5c), and after 5 min, the new sterilized IVC was transferred from the pass box to the WS (Fig. 5d). The IVCs that were in use were clearly separated from those that were not. When changing cages, a shelf unit was used to avoid contamination. The used and new cages were placed on the left and right sides of the bottom shelf of the shelf unit, respectively, while the used and new lids were placed on the left and right sides of the middle shelf of the shelf unit, respectively. The new cages were placed on the top shelf (Fig. 6a). The mice were moved to the IVC using forceps, taking care to prevent any physical contact between them. The forceps were disinfected by dipping them in 70% ethanol and exchanged for each cage (Fig. 6b). For the sterility tests, each mouse was held by hand to sample its feces. In addition, cage changes were performed once a week.

Evaluation 2

For the microbiome experiment, the duration for which the GF and GB mice could be maintained together without cross-contamination in a non-barrier room was tested. Mice with ASF were used as the GB mice. In this experiment, we used 6 IVCs (2 and 4 cages for GF and GB mice, respectively). The procedure for cage changes was performed as described in Evaluation 1. The first 3 IVCs exchanged contained GB mice, while the subsequent 3 IVCs exchanged contained GF, GB, and GF mice, respectively (Fig. 7). Outer gloves were replaced with fresh ones before changing the IVC to accommodate the GF mice.

Evaluation 3

For the BSL2 microbiome experiment, the duration for which GF mice could be maintained without becoming infected in a negative pressure-controlled Class II (Type A2) biosafety cabinet in an animal room, with cage changes, was tested. The breeding environment of the IVC and the bB were set up in a BSL2 animal room. Considering the general evaluation period for animal microbiome experiments, the target maintenance timeframe was 4 weeks. Two IVC cages were used in this experiment (Fig. 8) and the procedure for cage changes was performed as described in Evaluation 1.

In Evaluation 1–3, the time from when the worker entered the main room to the completion of cage changes was measured.

Cleaning procedures after operation

The bB, WS interior, work bench, and floor were dis-

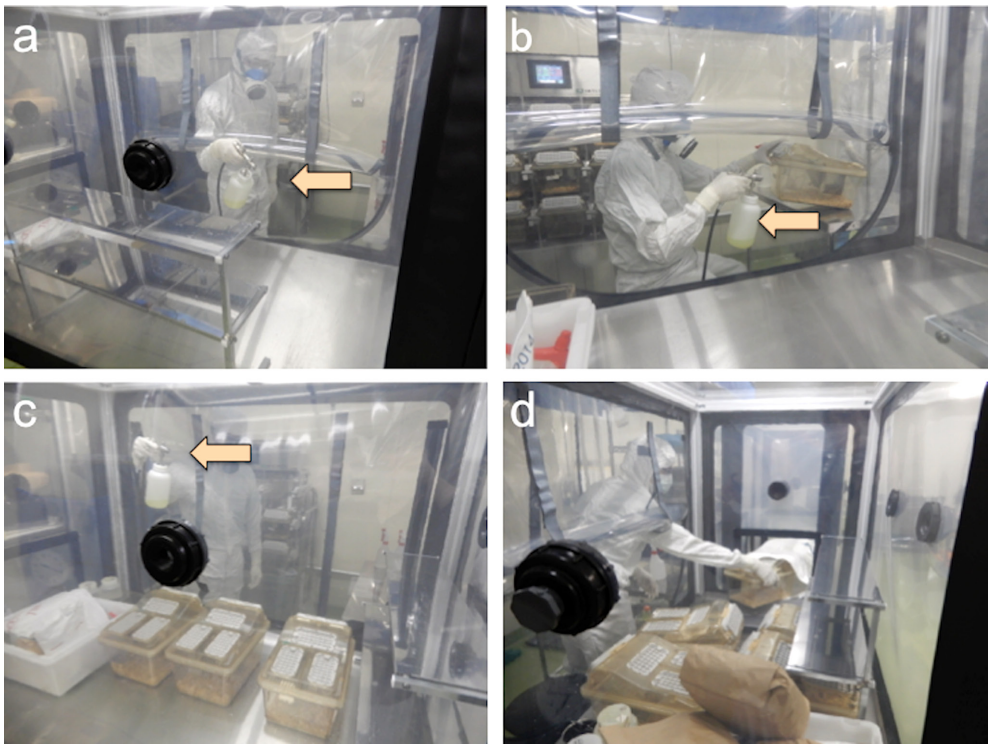

Fig. 5. The preparation for cage changes. (a) Disinfection of the working station (WS) was conducted before use. The arrow indicates the spraying of MB-10. (b) Disinfectant was applied when bringing the individual ventilation cage (IVC) in use into the WS. The arrow indicates the spraying of MB-10. (c) After that, the area in the WS was disinfected for 5 min before use. The arrow indicates the spraying of MB-10 from the zipper gap. (d) The sterilized equipment was transferred from the pass box into the WS. This WS can contain three in-use and three replacement cages. The procedure shown here was repeated when performing the cage change, regardless of whether they were for GF mice or not. This method was used in Evaluation 1 and Evaluation 2.

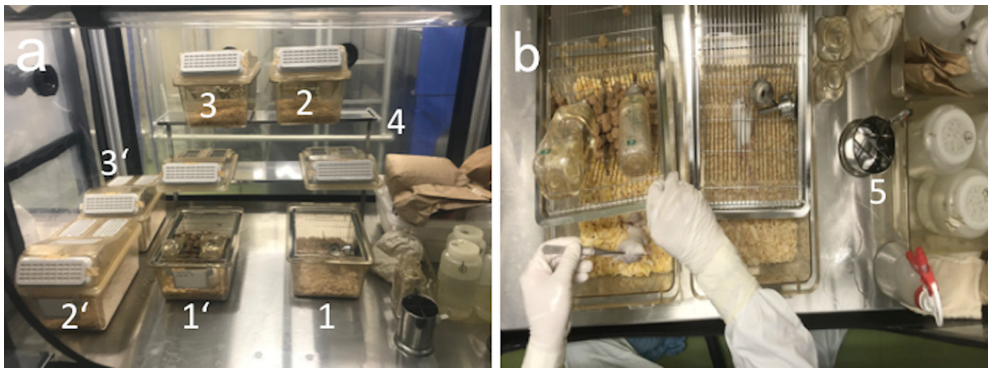

Fig. 6. Cage-change procedure in the working station (WS). (a) The WS contained three in-use cages (1'–3'), three replacement cages (1–3) and a shelf (4), which were arranged as shown. (b) The mouse was moved using forceps, which were soaked in 70% alcohol (5). Separate sets of forceps were used for each cage.

infected using 70% ethanol and a sterile towel. The floor in the exterior part of the bB was disinfected by mopping it with 200 ppm chlorine water.

Microbiological inspection of GF and GB mice

Sterility testing was performed at least once a month. Fresh feces and dirty bedding were collected and cultured in Semisolid Thioglycollate medium (Eiken Chemical Co., Ltd., Tokyo, Japan) at 37°C and room temperature for 2 weeks, respectively. They were also cultured in potato dextrose agar (PDA, Eiken Chemical Co., Ltd., Tokyo, Japan) at room temperature for 2 weeks. In addition, each IVCs was wiped using a swab, and the microorganisms on the swab were cultured on

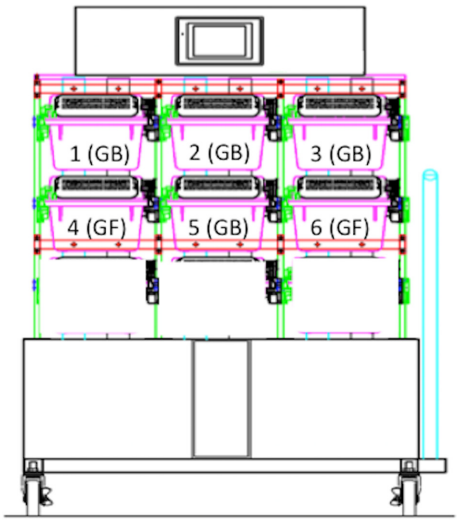

Fig. 7. Rearing experiments of germ-free (GF) and gnotobiotic (GB) mice in one rack. In Evaluation 2, the cage-change work was performed in the following order. Firstly, the three upper cages (1, GB; 2, GB; 3, GB) were changed, followed by the three lower cages (4, GF; 5,GB; 6, GF). The procedure shown in Fig. 5 was repeated when performing the cage changes, regardless of whether they were for GF or GB mice. Outer gloves were replaced with fresh ones before changing the individual ventilation cages for the GF mice.

Fig. 8. Cage-change procedure in the safety cabinet. The safety cabinet contained two in-use (1', 2') and two replacement cages (1, 2). Mouse transfer followed the procedure in Fig. 6b. This method was used in Evaluation 3.

PDA for 2 weeks [13].

Confirmation of microbial contamination in GB mice was performed at least once a month. Fresh feces were cultured on horse blood agar (Eiken Chemical Co., Ltd., Tokyo, Japan) and DHL agar (Eiken Chemical Co., Ltd., Tokyo, Japan) at 37°C for 2 days under aerobic conditions. They were also cultured on Brucella agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) with 5% horse blood at 37°C for 3 days under anaerobic conditions and on PDA at 37°C for 1 week under aerobic conditions. Microbial contaminants were identified by colony and cell morphology.

Results

Evaluation 1

GF mice were maintained for at least 12 weeks in the positive pressure-controlled conventional non-barrier animal room. To verify its long-term viabillity, breeding was continued up to 38 weeks. During this period, cage changes were performed 114 times, while sterility testing was performed 117 times (Table 1). Exchanging three cages took about 20 min to complete after setting them up the first time.

Evaluation 2

GF and GB mice could be bred in one IVC rack, without cross-contamination, for 12 weeks in the positive pressure-controlled conventional non-barrier animal room. To verify if this condition could be maintained during prolonged use, breeding was continued up to 29 weeks. During this period, cage changes, sterility testing, and ASF contamination checks were performed 174, 48, and 68 times, respectively. Three cage exchanges in two cycles took about 40 min to complete after the initial preparations.

Evaluation 3

GF mice could be maintained without infection for at least 12 weeks in the rearing environment within the negative pressure-controlled safety cabinet. To verify its reliability, breeding was continued up to 14 weeks. Dur-

Table 1. The results of microbiological inspection for germ-free and ASF mice

	Microbiological grade of mice	No. of cages	Implementation period (weeks)	No. of cage changes	No.		
					Sterility test	Confirmation of contamination	Positive
Evaluation 1	GF	3	38	114	117	ND	0
Evaluation 2	GF+GB	2+4	29	174	48	68	0
Evaluation 3	GF	2	14	28	32	ND	0

ND, not done.

ing this period, cage changes and sterility testing were performed 28 and 32 times, respectively. Two cage exchanges took about 15 min to complete after the initial preparations.

Discussion

There has been a recent increase in research interest in animal microbiome experiments. Several studies have been conducted on human feces to identify bacteria that affect the differentiation of intestinal lymphocytes [14, 15]. The efficient rearing and experimental environment that we achieved in this study using the IVC and bB can contribute to the efficient progress of research in this area.

In this study, all GF and GB mice were maintained stably without contamination. According to previous reports, GF and GB mice are normally bred in an IVC that is introduced into a barrier room [6, 8, 10]. The results of Evaluation 1 show that if using a conventional non-barrier animal room, one can easily perform animal experiments with GF or GB mice in an environment that combines a movable IVC rack with a bB that can be designed to accommodate any size.

Unlike a previous report [11] which stipulated that the WS and other related facilities be shared between two people, in our proposed setup, one person was able to perform all the operations. In addition, disinfection of the IVC could be switched from submersion of it in the sterilizing agent to spraying it with the sterilizing agent. We could also keep GF/GB mice in the IVC for a longer period. It was crucial to have sterilization conditions set using the actual temperature of the sterilization target (raising equipment, water, diet, etc.) by measuring the temperature with the data logger before the start of the experiment. We believe that it is important to include a data logger system in each facility.

In terms of VI operation, not only was the preparation time shortened but this system also did not require thick rubber gloves, which greatly improves the ease of rearing and performing experiments and makes it possible to perform delicate operations. For example, sharp injection needles that were previously avoided due to the possibility of air leak accidents can be used in this system. In addition, since the WS is spacious, it is possible to include a behavior analysis device, which could not be used previously, with appropriate sterilization treatments. This will greatly contribute to future animal experiments that use GF/GB mice. Furthermore, having an IVC per cage seemed to reduce the risk of damage caused by microbial contamination compared with having a VI in several cages.

In Evaluation 2, GF and GB mice were bred at the same time in one IVC rack. We speculated that there was a risk of contamination when opening the lid of the GF mouse cage following work performed in the GB mice cage. However, both the GF and GB mice were stably maintained without contamination. Furthermore, the ventilation frequency of the WS used in this system was set at 100 or more exchanges per hour. In other words, the air inside the WS was refreshed every 36 seconds. In cage exchanges, it took a minimum of 1 min between closing the lid of the previous cage and opening the lid of the next cage. Therefore, it was considered that the air in the WS was replaced with clean air before the next cage was opened. Microbiome animal experiments using VIs have required the use of one VI per test group. The abovementioned results suggest the possibility of reducing the space required to the size of an IVC compared with a VI. Similarly, the preparation time was shorter than that using a VI as well. In this study, we evaluated ASF mice in which eight strains of bacteria had colonized. However, more validation tests using other strains of bacteria are recommended for future work.

When using BSL2 bacteria, a negative pressure environment, breeding room, and a negative pressure environment safety cabinet were deemed necessary to protect the workers. Evaluation 3 was designed to mimic an animal laboratory in which BSL2 bacteria or feces are administered into mice. It is highly possible that environmental bacteria would flow into the negative pressure-controlled safety cabinet installed in the rearing room through the air introduced from outside. Therefore, we thought it might be difficult to rear the GF mice under these conditions. However, it was confirmed that the GF mice could be maintained in this system, in which all cabinets were enclosed in the bB. This system could contribute to the precision required in a seeding experiment that uses BSL2 microbes, by suppressing the contamination caused by environmental bacteria. Based on this study, we proposed a new experimental environment that can be used to efficiently carry out animal microbiome research in animal facilities.

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