Experimental Animals
Exp. Anim. 71(1), 28–35, 2022

Original

Eliminating murine norovirus, *Helicobacter hepaticus*, and intestinal protozoa by embryo transfer for an entire mouse barrier facility

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Abstract: Pathogens can affect physiological and immunological reactions in immunocompromised animals and genetically engineered mice. Specifically, murine norovirus (MNV), *Helicobacter*, and intestinal protozoa are prevalent in rodent laboratory facilities worldwide. In this study, microbiological test results of the soiled bedding of sentinel mice showed the prevalence of MNV (50.9%, 28/55), *Helicobacter hepaticus* (29.1%, 16/55), *Trichomonas* spp. (14.5%, 8/55), and *Entamoeba* spp. (32.7%, 18/55). No single infections were detected as all cases were confirmed to have complex infections with two or four pathogens. In previous studies, the success rate of the cross-fostering method was not perfect; therefore, in this study, the entire mouse strain of the SPF rodent facility was rederived using embryo transfer. For up to three years, we confirmed that the results were negative with regular health surveillance tests. Embryo transfer was, thus, determined to be an effective method for the rederivation of specific pathogen free (SPF) barrier mouse facilities. This is the report for the effectiveness of embryo transfer as an example of successful microbiological clean-up of a mouse colony with multiple infections in an entire SPF mouse facility and embryo transfer may be useful for rederiving.
Key words: embryo transfer, *Entamoeba* spp., *Helicobacter hepaticus*, murine norovirus, *Trichomonas* spp.

Introduction

Pathogens can affect physiological and immunological reactions in immunocompromised animals and genetically engineered mice. Specifically, murine norovirus (MNV), *Helicobacter hepaticus*, and intestinal protozoa are prevalent in rodent laboratory facilities around the world. Of these, MNV has been identified as the most prevalent pathogen in laboratory mouse facilities. MNV can affect immune function in normal mice, but in immunodeficient mice with a deficiency of STAT1 and IFN receptors, it may be fatal [1–6]. In laboratory mouse facilities, *Helicobacter* spp. infections have been identified worldwide and are associated with the occurrence of inflammatory bowel disease (IBD), hepatitis, and liver and intestinal carcinomas.

Furthermore, immunocompromised mice such as *Rag1/Rag2* and *Pkrdc*^{scid} mice have been reported to be highly affected by *Helicobacter* spp. [2, 7–15], *Trichomonas muris* infections affect the homeostasis of mucosal immune cells and susceptibility to colitis in mice [16, 17]. In our laboratory mouse facility, more than 90% of the mice are genetically modified and immunocompromised. Complex infections of MNV, *Helicobacter hepaticus*, and intestinal protozoa, which are the main pathogens affecting metabolic diseases, immune studies, and tumor experiments such as colon cancer xenograft studies, were confirmed at our facility. In accordance with animal welfare principles, pathogen control also provides refinement of the animal protocol by reducing

(Received 4 February 2021 / Accepted 15 July 2021 / Published online in J-STAGE 30 August 2021)
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the pain and distress of the animals [18]. Rederivation of infected mice is consistent with ensuring the scientific results of animal protocols and the pursuit of animal welfare (3R).

In previous studies, cross-fostering was used to clean up complex infections in mice, but this strategy did not eliminate MNV [4, 19, 20]. While in previous studies using embryo transfers, there was no report on whether the removal of intestinal protozoa (*Entamoeba* spp.) was successful [21–23]. This study aimed to confirm the effectiveness of the embryo transfer cleaning method for combined mouse MNV, *Helicobacter*, and intestinal protozoa (*Trichomonas* spp., *Entamoeba* spp.) infections.

Materials and Methods

Animals

CD1 mice (Outbred Crl: CD1 (ICR), 4weeks) were imported from OrientBio (Seoul, Korea) and then bred under the strictly regulated conditions of the barrier facility. Due to their high reproductive performance, CD1 mice were used as recipients for the rederivation of genetically engineered mice. Various genetically engineered mice have been introduced to our facility from global commercial vendors and research institutes. Mice were housed 5 per cage in a room maintained at 23 ± 1°C with an average relative humidity of 40–60%, under a 12:12-h light: dark cycle. Mice were housed in individually ventilated caging (Thoren, Hazleton, PA, USA). Mice had access to irradiated mouse feed (LabDiet 5053, USA) and autoclaved reverse osmosis water *ad libitum*. Each cage contained autoclaved aspen bedding (Woojung, Suwon, Korea), and synthetic nesting material (Ancare, Bellmore, NY, USA). Sterilized forceps that were briefly dipped in disinfectant (Vircon-S, Lanxess, Köln, Germany) were used during weekly cage changes, when the mice were moved from dirty to clean cages.

Animal protocol

The Institutional Animal Care and Use Committee (IACUC) of the Sungkyunkwan University School of Medicine (SUSM) reviewed and approved this study. The SUSM is an Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) accredited facility and is guided by the Institute of Laboratory Animal Resources (ILAR).

Facility

The Center for Laboratory Animal Research of SUSM is an AAALAC-accredited institution. Our facility consists of nine mouse rooms with individually ventilated caging systems. Transgenic and knockout strains are bred in the SPF barrier. All mice are sourced from approved vendors and quarantined before entry into the main mouse colony. Mice from the research institute were rederived by embryo transfer.

Cross-fostering

In cross fostering, heterozygotes were used for both male and female. A hysterectomy of the pregnant dam was performed at 19 days aseptically, after dipping the uterus in 10% Tego solution (Alkyldiaminoethyl- glycine HCl 5% Sungkwang, Anyang, Korea), and all the pups were moved to a pathogen-free CD-1 surrogate mother in the Bio-safety cabinet with sterilized forceps. All surgical instruments, including two pairs of scissors, were autoclaved. After povidone dressing, a skin midline incision was made through the skin, using sterile scissors. The skin incision should be extended from the xiphoid process to the inguinal area. Cuts were then made gently through the uterine wall with a second pair of scissors to avoid cross-contamination from the skin of the mother to the sterile uterus and pups. The surrogate mother was moved to a separate area. Health surveillance was conducted for the surrogate mother and one pup in the litter.

Embryo transfer

In embryo transfer both male and female were heterozygote or wild type females and heterozygote males were used. 8weeks female mice were administered 5 IU PMSG (PMSG, Sigma Chemical Co., Steinheim am Albuch, Germany) intraperitonially. After 48 h 5 IU hCG (Sigma Chemical Co.) was administered to female mice intraperitonially and female mice were mated with male mice naturally. At 17 h after hCG injection, female mice were sacrificed by cervical dislocation, and their oviducts were quickly collected and transferred to a fertilization dish covered with paraffin oil. Under microscopic observation, cumulus oocytes complexes were collected from the oviducts and transferred to a 200-μl drop of fertilization medium (M2 medium, Sigma Aldrich, Steinheim am Albuch, Germany). The number of ovulated oocytes and fertilization ability of oocytes in each group were examined. Pseudo-pregnant female mice were distinguished by the presence of a copulation plug after mating with vasectomized males. These females were anesthetized with subcutaneous injections of alfaxalone (80 mg/kg; alfaxan, Jurox, Rutherford, Australia) and xylazine (10 mg/kg; Rompun, BAYER KOREA Ltd., Seoul, Korea). Embryo transfer into the infundibulum, opening end of the oviduct, was performed. Each recipient received 20–25 two-cell embryos. Embryo transfer was

performed only when the ampulla was swollen, and the reproductive tract had good blood circulation. Surrogate mothers which were past their due date had a cesarean section within 24 h in four mouse strains. The recipient females were moved to separate areas. For analgesic support, they received meloxicam subcutaneously (5 mg/kg; Metacam, Boehringer Ingelheim, Rhein, Germany). The recipient female mice recovered at 37°C on a heating pad until they were alert and spontaneously moveable. They were kept in a cage until the offspring were born, after which they took care of their pups when they weaned. Health surveillance was conducted for the surrogate mother and one pup in the litter.

Sentinel program

Sentinel program utilizes exposure of soiled bedding to sentinel (BALB/c, 4 weeks, female), imported from OrientBio (Seoul, Korea). Bedding samples from several cages are placed into the sentinel cage for three months. Each room has four to six individually ventilated caging racks attached sentinel cage a rack and two sentinels were housed in cage. Regular health surveillance for sentinel mice was performed every three months.

Pathogen test

Both PCR and serological tests were conducted in accordance with the manufacturer's instructions to assess the prevalence of MNV. Serological testing for MNV was performed using an ELISA kit (Charles River Laboratories, Wilmington, MA, USA). The MNV primers were as follows: Primer 1F (5'-GCC ATG CAT GGT GAA AAG-3'), Primer 1R (5'-CAT GCA RAC CAG GCG CAT AG-3'), Primer 2F (5'-ACA RTG GAT GCT GAG ACC-3'), and Primer 2R (5'-CAA CCA CCT TGC CAG CAG-3') [24]. RT-PCR–based testing was performed using feces freshly collected from the live mice or after euthanasia of the mice. RNA was extracted from the supernatant of the feces homogenized in sterile water using an RNA purification kit (QIAamp Viral RNA Mini Kit, QIAGEN, Hilden, Germany). Purified RNA was reverse-transcribed and amplified using specific primers and a Maxime RT-PCR PreMix Kit (iNtRON Bio. Seongnam, Korea).

Helicobacter hepaticus testing was performed using PCR, and the primers were as follows: B38 (5'-GCATTT GAA ACT GTT ACT CTG-3') and B39 (5'-CTG TTT TCA AGC TCC CC-3') [25]. DNA was extracted from the cecum contents and stool with a DNA purification kit (QIAamp Viral DNA Mini Kit, QIAGEN). Gene of *Helicobacter hepaticus* was amplified with premixure taq polymerase (iNtRON Bio.).

Intestinal protozoa were diagnosed through the examination of the fresh feces or direct smears of the intestinal contents.

Results

Microbiological tests were performed for 55 sentinel mice in our animal facility using their soiled bedding. Sentinel mice were placed in six of the nine rooms. The two rooms were empty for transferring rederived mice. The remaining room was used as an isolation space for animal testing, and there were no mice that required additional rederiving. There were multiple individually ventilated caging systems in the room, each with one sentinel cage per side. The fifty-five sentinel mice were used to conduct pathogen tests by room (Table 1). In case the sentinel mice identified positive for each pathogen, the mouse strains housed in the room were rederived.

The prevalence of MNV (50.9%, 28/55), *Helicobacter hepaticus* (29.1%, 16/55), *Trichomonas* spp. (14.5%, 8/55), and *Entamoeba* spp. (32.7%, 18/55) were determined. For each pathogen, there were no single infections, and all cases were confirmed to have two to four complex infections (Fig. 1). Of the 58 rederived mouse strains, nine were immunocompromised and infected with all four pathogens. Two or all four pathogens were infected with other mouse strains, including immunocompetent genetically engineered mouse (GEM) and a wild type. Most immunocompromised GEM were found to have rectal prolapse and diarrhea. The detailed list of mouse strains are provided in Table 2.

In this study, two methods, cross-fostering and embryo transfer, were used to rederive mice infected with MNV, *Helicobacter hepaticus*, and intestinal protozoa (*Trichomonas* spp., *Entamoeba* spp.). The efficacy of cross

Table 1. Prevalence of pathogen in sentinel mice by room

Pathogen prevalence	Room 1	Room 2	Room 3	Room 4	Room 5	Room 6	Total
MNV	6/9[a)]	4/9	6/9	4/9	4/9	4/10	28/55
Helicobacter	4/9	4/9	4/9	4/9	0/9	0/10	16/55
Trichomonas spp	4/9	4/9	0/9	0/9	0/9	0/10	8/55
Entamoeba spp	6/9	4/9	0/9	0/9	4/9	4/10	18/55

[a)]Prevalence of pathogen in sentinel mice (infected mouse number/tested mouse number)

 doi: 10.1538/expanim.21-0026

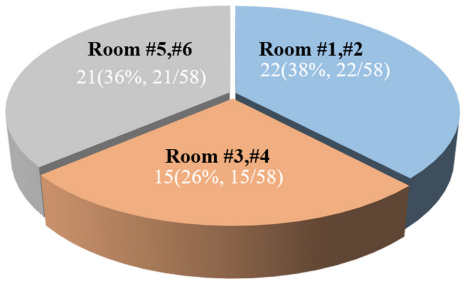

Fig. 1. Prevalence of complex infection in mouse strains. Infection status of room is represented by colors. Twenty two mouse strains in room #1 and #2 were co-infected with MNV, *Helicobacter hepaticus*, *Trichomonas* spp., *Entamoeba* spp. (Blue) Fifteen mouse strains in room #3 and #4 were co-infected with MNV, *Helicobacter hepaticus.* (Orange) Twenty one mouse strains in room #5 and #6 were co-infected with MNV, *Entamoeba* spp. (Gray).

fostering for MNV performed in this study was 91.7% (11/12). While with the rederivation method via embryo transfer resulted in 46 mouse lines being successfully cleared of the pathogens (Table 3). In two mouse strains, all newborn mice were cannibalized by the surrogate mother after cross-fostering. One mouse strain (C57BL/6 -Hdac1$^{tm1.1Mrl}$/Tac) was positive for MNV in a post-rederivation (cross-fostering) health surveillance test (Table 2). After hysterectomy, the pups were considered to have been infected with MNV during the transfer of the newborn mouse to the surrogate mother. This mouse strain was successfully rederived by embryo transfer. Embryo transfer was more effective for clearing multiple pathogens.

Regular health surveillance tests were conducted using the sentinel program. Follow-up health surveillance tests for MNV, *Helicobacter hepaticus*, and intestinal protozoa (*Trichomonas* spp., *Entamoeba* spp.) were negative for up to three years.

Discussion

The prevalence of four pathogen infections in our animal facilities differed slightly from those in previous studies. The microbiological infection status of laboratory mouse facilities has been reported to vary by country and type of animal facility (university, research institute, animal vendor, pharmaceutical company). In our animal facility, the MNV infection rate was 50%, higher than that previously reported by Henderson (32.4%), Yeom (36.5%, Serology), Hayashimoto (15%, Serology, 1.97%, PCR) [1, 4, 8]. In Korea, the rate of MNV infection was high in the genetically modified mouse group in the production facility (6.6%), in the breeding animal population (9.6%), and the genetically modified mouse group (27%) [26].

In Hayashimoto study, the infection rates in the laboratory animal facilities (9.38%) of the universities and research institutes had been confirmed to be lower than in pharmaceutical companies and CRO laboratory animal facilities (23.8%) [1]. In our facility with more than 90% GEM, the incidence of MNV infection was reported to be higher than in previous domestic studies. It was considered that differences in sensitivity depending on the MNV test method and differences in infection rate depending on the characteristics of the area and animal facility could occur. In our animal facility, the *Helicobacter hepaticus* infection rate was 29.1%, which was lower than that previously reported in the Yeom study (36.5%, PCR), but it was much higher than that of Hayashimoto study (3.17%, PCR), and there were large regional differences [1, 4]. The infection rate of our animal facility for *Trichomonas* spp. (14.5%, microscopy) differed from that of the laboratory animal facilities in Korea (34.9%, microscopy, Won 2010) and Hayashimoto study (4.95%), and the infection rate of *Entamoeba* spp. (32.7% microscopy) was confirmed to be higher than that of laboratory animal facilities in Korea (27.9%, microscopy) and Hayashimoto study (8.49%, microscopy) [1, 27]. The prevalence of pathogens in animal facilities may be underestimated or overestimated by region, depending on the time and method of testing for each pathogen, the number of tests, and the status of previous infections. Despite these limitations, it can be used as a useful indicator of which pathogens are at high risk in animal facilities [6]. Most of immunocompromised GEMs which were complex infected showed clinical symptoms such as rectal prolapse and diarrhea in this study. But it was unexpected that no clinical sign was identified in both immunocompetent GEMs and wild-type mice which were multi pathogen infected. There was no difference in clinical signs between immunocompetent GEMs and wild-type mice. It is unclear whether such complex infections had a mutual effect between pathogens, opportunistic infections, or differences in susceptibility to the immune status of the mice. Further research is needed to confirm the cause of the complex infections.

In previous studies, the efficiency of MNV rederivation through cross-fostering was 78.2–94% (Yeom 78.2%, Buxbaum 94%) [4, 19, 20]. MNV can be in the form of a fomite and has high external viability and is thought to be transmitted via skin infection to the newborn mice through the external environment. A previous study also reported infections of this kind [20]. Although MNV infection does not cause clinical symptoms in mice, combined MNV and *Helicobacter* spp. infections have been reported to accelerate the progression of

Table 2. Post-rederivation Health surveillance result in mouse strains

Room	No.	Strain	Immune status[a)]	MNV		Helicobacter		Trichomonas spp.		Entamoeba spp.		No. of transferred embryos	Total No. of pups	Method
				Surrogate mother	Pup	Surrogate mother	Pup	Surrogate mother	Pup	Surrogate mother	Pup			
1	1	B6.mu$^{-/-}$	lack B cell	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	7	Embryo transfer
1	2	B6.Rag$^{-/-}$	lack B, T cell	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	60	20	Embryo transfer
1	3	B6.P4$^{-/-}$	decrease	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	40	16	Embryo transfer
1	4	B6.TT$^{-/-}$	CD4+ T cell decrease	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	6	Embryo transfer
1	5	B6.C3$^{-/-}$	CD4+ T cell lack complement	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	40	7	Embryo transfer
1	6	B6.CD5$^{-/-}$	lack CD5 T cell	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	6	Embryo transfer
1	7	B6.CD45.1$^{-/-}$	Different allotype to B6	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	7	Embryo transfer
1	8	B6.SAP$^{-/-}$	lack NK T cell	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	40	12	Embryo transfer
1	9	B6.CD1d$^{-/-}$	lack NK T cell	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	60	18	Embryo transfer
1	10	C57BL/6	Normal	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	60	16	Embryo transfer
		-Hdac1$^{tm1.1Mrl}$/Tac		0/3	*3/3	0/3	0/3	0/3	0/3	0/3	0/3		14	Cross-fostering : MNV Infection
1	11	B6.129SV. HDAC2$^{-/-}$	Normal	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3		15	Cross-fostering
1	12	B6.SIK1$^{-/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	40	11	Embryo transfer
1	13	B6.Mtor$^{f/f}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	5	Embryo transfer
1	14	B6.GFP -LC3$^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	3	Embryo transfer
1	15	B6.Dscr1$^{-/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	40	10	Embryo transfer
2	16	B6.Ssu72$^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	6	Embryo transfer
2	17	B6.Smek2$^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		8	Cross-fostering
2	18	B6.Alb Tg	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	5	Embryo transfer
2	19	B6.Ap2 Tg	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2		10	Cross-fostering
2	20	B6.Prmt1$^{-/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	40	9	Embryo transfer
2	21	B6.Ap2 Tg	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	40	11	Embryo transfer
2	22	B6.Peli1$^{-/-}$	Normal	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	60	20	Embryo transfer
3	23	B6.Smek$^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		9	Cross-fostering
3	24	B6.MAGI3 $^{-/-}$	Normal	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	60	21	Embryo transfer
3	25	B6.3xAD Tg	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		9	Cross-fostering
3	26	B6.Rock1$^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	10	5	Embryo transfer, Cesarean section
3	27	B6.Nas Tg	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2		15	Cross-fostering
3	28	B6.Mdx $^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	10	6	Embryo transfer
3	29	B6.Nrf2$^{-/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	20	13	Embryo transfer
4	30	B6.Hif1αTg	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	10	7	Embryo transfer
4	31	B6.Pink1 Tg	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	10	6	Embryo transfer
4	32	B6.Pin1$^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	10	5	Embryo transfer
4	33	B6.Galectin9 Tg	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	10	6	Embryo transfer
4	34	B6.Cnb$^{-/-}$	Normal	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	60	12	Embryo transfer
4	35	B6.Ts65 Tg	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	6	Embryo transfer
4	36	B6.Pdx1 Tg	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	7	Embryo transfer
4	37	B6.Rip Tg	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	8	Embryo transfer
5	38	B6.Pelino1 Tg	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	40	10	Embryo transfer
5	39	B6.Bex4$^{-/-}$	Normal	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	60	16	Embryo transfer
5	40	B6.LKB1$^{f/f}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	4	Embryo transfer, Cesarean section
5	41	B6.Prmt1$^{+/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	8	Embryo transfer
5	42	B6.Ssu72$^{+/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	6	Embryo transfer
5	43	C57BL/6	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	8	Embryo transfer
6	44	B6.Pdx1$^{-/-}$Dscr1$^{-/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	20	9	Embryo transfer
6	45	B6.GFP Tg	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	8	Embryo transfer
6	46	B6.KRAS$^{-/-}$Dscr1$^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	8	Embryo transfer
6	47	B6.P16 Tg	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	3	Embryo transfer
6	48	B6.Cdo$^{-/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	20	10	Embryo transfer
6	49	B6.Boc$^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	5	Embryo transfer, Cesarean section
6	50	Tg(Fos-lacZ)34Efu/J	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2		13	Cross-fostering
6	51	B6.TCR$^{-/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2		14	Cross-fostering
6	52	B6.TCF4 $^{+/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	20	8	Embryo transfer
6	53	B6.CREBH $^{+/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2		13	Cross-fostering
6	54	B6.P53$^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1		7	Cross-fostering
6	55	B6.S6K2$^{-/-}$	Normal	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	60	3	Embryo transfer, Cesarean section
6	56	B6.Prmt7$^{-/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2		10	Cross-fostering
6	57	B6.S6K1$^{-/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2		11	Cross-fostering
6	58	B6.S6K1$^{-/-}$S6K2$^{-/-}$	Normal	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	40	12	Embryo transfer

[a]Nine mouse strains (No.1–9) were immunodeficient among 58 mouse strains, and other mouse strains (No.10–58) were immunocompetent. "+" shows positive and "–" shows negative.

 doi: 10.1538/expanim.21-0026

Table 3. Efficacy of rederivation method for MNV, Helicobacter, and Intestinal protozoa

Method	Efficacy	Prevalence of pathogen after rederivation			
		MNV	Helicobacter	Trichomonas spp.	Entamoeba spp.
Cross fostering (14 Mouse Strain)	91.70% 11/12[a)]	12.50% 3/24[b)]	0% 0/24	0% 0/24	0% 0/24
Embryo transfer (46 Mice strains)	100% 46/46	0% 0/94	0% 0/94	0% 0/94	0% 0/94

[a)]Efficacy is calculated as a percentage (rederived mouse strain number / infected mouse strain number). The overall number of cross-fostered mouse strains was 14, all pups were cannibalized by surrogate mothers in two stains, which were excluded. [b)]Prevalence of pathogen after rederivation (infected mouse number/tested mouse number), MNV infected pups (B6.129SV.HDAC1$^{-/-}$) were identified.

Helicobacter-related IBD in Mdr1a-null mice [12]. To remove MNV from the body, the acquired immune system is essential. In mice with a deficient acquired immune system, MNV does not cause clinical symptoms but can affect them as an animal carrier of the virus.

A previous study has indicated that embryo transfer was used to successfully eradicate viruses, bacteria, and parasites (pinworms: *Syphacia*) in mice and it was expected that it could be effective for other pathogens [23, 28, 29]. This study confirmed that viruses, bacteria, and intestinal protozoa (*Trichomonas* spp., *Entamoeba* spp.) can also be successfully rederived by embryo transfer. In a previous study, antibiotic treatment was successful after cross-fostering for *Helicobacter* infection, but it was difficult to apply to mice with complex infections in our animal facility. This is because, unlike *Helicobacter* spp., MNV and intestinal protozoa do not have any therapeutic effect on antibiotics.

Considering the cases of mouse pathogen elimination conducted in this study and previous studies, taking into account the aspects of mouse pathogen infection in the animal facility, the fertility of individual mice, and the animals available (sex, age, number of mice), it is important to choose the appropriate method of rederivation. Embryo transfer is difficult to apply if mice have low fertility or are less responsive to exogenous hormone treatment for super-ovulation (such as A/J) and fertilized eggs cannot survive in a laboratory culture environment [30]. Cross-fostering via hysterectomy is recommended in this case, and care must be taken to prevent infection during surgery with thorough disinfection and sterilization. There are several considerations in the application of cross-fostering. Intensive care should be taken to avoid newborn mice during the hysterectomy process. If there is no thorough disinfection and control of infectious diseases, newborn mice should be managed so that they are not infected with the same MNV pathogen during the cross-fostering process. Moreover, if the surrogate mother cannibalizes the newborn mouse or does not care, the purpose of cleansing will be difficult to achieve. Cross-fostering is recommended for mice with a poor response to superovulation treatment or embryos that are poorly divided in the *in vitro* environment. It has the benefit of allowing only one female of the desired genotype to be used. The pathogen may not be completely eradicated in viral infections like MNV. Embryo transfer can be used successfully to eliminate all pathogens in mice, but it is difficult to use in mice that do not respond to superovulation treatment or *in vitro* culture. To collect embryos for embryo transfer, at least four females of the desired genotype are needed. Personnel with embryo transfer surgery expertise are required. Pathogen testing after rederiving is important in both methods to confirm that the target pathogen has been removed. In previous studies, there were some differences in timing and method of testing for each pathogen, depending on the method of rederivation. In the case of MNV, serology or PCR tests have been confirmed. In cross-fostering, serum tests were performed on newborn mice at 4–12 weeks of age and PCR tests were performed at 3–8 weeks of age [4, 19, 23]. In this study, MNV serological tests were performed for 6–8 weeks. The PCR test has a higher sensitivity than that of the serum test and can be tested earlier. In a serological test, a positive seroconversion result may be obtained based on the age of the newborn mouse, which means that it is necessary to proceed with the test at an appropriate time. Therefore, early PCR-based testing was determined to be a more appropriate method. Moreover, there is also a limitation, as serum tests cannot be carried out on animals with defective B cell-related immune functions. The advantage of PCR testing is that it confirms whether or not there is an infection before seroconversion, and the time that infected mice are kept in animal facilities and possibly spreading the infection, can be reduced. PCR was used in this study to test for 4–5 weeks of age. In the case of *Helicobacter*, newborn mice were tested by PCR at 4–8 weeks of age in a previous study, and the same 4–5 weeks of age were tested in this study [4, 19]. Mice have recently been microbiologically tested by RT-qPCR using dust collected by filters in indi-

vidual ventilation cages. In the case of MNV, this RT-qPCR method has been reported to be more sensitive than serum testing [31]. PCR testing of the exhaust air dust from the filters in individually ventilated cages can be helpful to improve the accuracy of the test.

Regarding animal welfare, cleaning mice infected with the pathogen can reduce the clinical symptoms, pain, and stress associated with the infection. This may reduce the distress of animals in animal protocols and prevent an increase in the number of animals used. We used four superovulated egg donors in the embryo transfer process. 10–20 females are required for IVF and ICSI procedures [23]. In cross-fostering process multiple litters of infected and SPF mice must be synchronized for hysterectomy. Some pups from infected litters do not survive the procedure and pups are sacrificed from SPF litters when they are replaced by cross fostered pups. Additionally, mice used in animal experiments must be used in a state that is free of specific pathogen infection, which best reflects normal physiological changes. In the case of the embryo transfer method, the number of animals used in comparison with the hysterectomy cross-fostering method is smaller on average, and the risk of newborn mice being infected with the pathogen during the process is low.

There is also less risk that the newborn mouse will not be taken care of by the surrogate mother. The cleaning process using embryo transfer may be viewed as a more humane method of rederivation than cross-fostering, if embryo transfer is feasible when considering the fertility of mice.

To promote the rederivation of an entire animal facility, several issues should be considered. It is necessary to agree on the progress of the rederiving process through communication with researchers. It is important to explain that the progress of pathogen cleaning may have an impact on the animal protocols of the researcher and that the reliability of the animal experiments is ensured by rederivation. Physical separation of surrogate mothers with new cross-fostered or embryo transferred and pathogen-tested mice during cleaning is ongoing to block re-infection should be managed. Management of housing, disinfection of space, and sterilization of articles is also important. When entering the clean area, gloves were overlapping and disinfection was added. Also the used housing products were separated and treated according to their location. Furthermore, it is important to maintain the designation of the person in charge by area, comply with access procedures according to the state of microbial infection, use disposable personal protective equipment, sterilize spaces, autoclave stuff, and sterilize with hydrogen peroxide fumigation. External animals from animal vendors should be imported only when no specific pathogen is present and they are cleaned by embryo transfer at external research facilities, such as universities and research institutes, where animals are brought in. Microbiological test was performed quarterly for sentinel mice and semi-annually for resident mice. This obligatory conversion must be carried out. To prevent re-infection after the completion of the cleaning of animal facilities, it is important to have an external animal import policy. Maintaining improved precautions after rederivation of the entire facility will help to prevent reinfection and, ultimately, will lead to animal welfare.

Conclusion

The prevalence of MNV (50.9%), *Helicobacter hepaticus* (29.1%), *Trichomonas* spp. (14.5%), and *Entamoeba* spp. (32.7%) were determined. For each pathogen, there were no single infections, and all cases were confirmed to have two to four complex infections. The clinical symptoms such as rectal prolapse and diarrhea was observed in the most of immunocompromised strains, but not in the immunocompetent GEMs and wild-type mice. Using embryo transfer, MNV, *Helicobacter hepaticus*, *Trichomonas* spp., and *Entamoeba* spp. were successfully removed. Cross-fostering can be used in mice for rederivation, when they cannot be transferred to the embryo. However this method did not completely eliminate the pathogen for MNV infections. In the case of mice with complex-pathogen infections, we confirmed that embryo transfer was effective even in the case of mice infected with complex pathogens. Three years have passed since the rederiving of our animal facility was completed, and no infection with the four pathogens has been confirmed to date. This is the report for the effectiveness of embryo transfer as an example of successful microbiological cleanup of a mouse colony with multiple infections in an entire SPF mouse facility and embryo transfer may be useful for rederiving other laboratory rodent facilities.

Funding

SBRI (Samsung Biomedical Research Institute) provided the funding of this study [SA0000].

Acknowledgments

The authors wish to thank the expert animal care of our staff throughout this study.

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