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The Use of Cross-foster Rederivation to Eliminate Murine Norovirus, *Helicobacter* spp., and Murine Hepatitis Virus from a Mouse Colony

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Over 10 mo, 287 mouse litters were cross-fostered by using 1 of 2 paradigms to eliminate murine norovirus (MNV), *Helicobacter* spp., murine hepatitis virus (MHV), and *Syphacia obvelata*. Paradigm 1 involved cross-fostering litters at younger than 48 h with no attention to the changing of bedding material. Paradigm 2 involved cross-fostering litters at younger than 24 h from cages in which the bedding material was changed within 24 h before cross-fostering. After cross-foster rederivation, mice were tested for the presence of *Helicobacter* spp. by means of fecal PCR at 4, 8, and 12 wk. Surrogates also were tested for MNV by use of multiplex fluorometric assay serology at 4 wk and fecal PCR at 12 wk. Surrogate mice were tested for MHV by means of MFIA at 4 wk and for pinworms by perianal tape test and fecal flotation at 4 and 12 wk. Compared with those from paradigm 1, litters from paradigm 2 were less likely to be positive for MHV and *Helicobacter* spp. The use of cross-foster rederivation alone was unsuccessful for the elimination of *Syphacia obvelata*. For cross-foster rederivation, we recommend that litters be younger than 24 h and from cages in which the bedding material was changed within 24 h before cross-fostering. The presence of MNV, *Helicobacter* spp., and MHV can be predicted reliably at 12, 8, and 4 wk, respectively.

Abbreviations: MHV, murine hepatitis virus; MFIA, multiplex flurometric assay; MNV, murine norovirus

The elimination of rodent pathogens is desirable from both animal welfare and scientific perspectives. Rodents that harbor murine norovirus (MNV), *Helicobacter* spp., and murine hepatitis virus (MHV) can affect animal welfare by causing clinical disease.[4,5,33,34] In addition, subclinical infections can affect welfare by increasing the variability of experiments and confounding results, which can increase the number of animals used. More specifically, MNV can cause histopathologic changes[24] that confound experimental data. *Helicobacter* species such as *H. hepaticus* or *H. bilis* can confound carcinogenesis studies and alter inflammatory responses in infected mice.[13,23,28] Murine hepatitis virus can cause immunosuppression, blood dyscrasias, and increased tumorcidal activity of macrophages.[6,25,26,32] *Syphacia obvelata* can inhibit the development of diabetes in NOD mice, terminate self tolerance and enhance autoimmune disease, and stimulate mucosal immunity.[1,2,14]

Rederivation of mice can be accomplished by embryo transfer, hysterectomy of late-term fetuses, or by cross-fostering neonatal pups to surrogate mothers with the appropriate microbial status. The primary advantage of using cross-fostering as a means of rederivation is that it is less invasive and technically demanding than embryo transfer. Furthermore, it does not require the euthanasia of donor females. Cross-foster rederivation should be considered where insufficient adult mice or embryos are available. A disadvantage of using cross-fostering as a means of rederivation is the increased opportunity for neonates to become contaminated after they are born. Moreover, cross-foster rederivation will not prevent contamination of a litter if the microbial agent undergoes intrauterine transmission. Neonatal cross-foster rederivation is reported to be effective in eliminating *Helicobacter* spp.[10,12,30,31,35] and MHV;[22,35] however, the literature lacks descriptions of its utility in the elimination of MNV and pinworms. In this study, we assessed the feasibility of using cross-foster rederivation as a means to eliminate not only *Helicobacter* spp. and MHV but also MNV and pinworms from a mouse colony where these agents are enzootic.

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Materials and Methods

Facility. The Biologic Resources Laboratory is the central animal facility at the University of Illinois at Chicago, an AAALAC-accredited institution. The first floor of the facility is 32,705 ft^2 and housed both conventional and barrier mice. At the initiation of the study, conventional and barrier mice were housed in separate and distinct areas of the facility.

Husbandry. Before the rederivation program, conventional mice at the facility were housed in open, nonsterile, sanitized shoebox cages. The cages were changed on an open bench; dirty cages were disassembled and stacked into component parts in the room. Mice were fed nonirradiated, nonautoclaved diet (rodent diet 8640, Harlan Teklad, Madison, WI), offered municipal water in bottles, and were housed on nonsterilized corncob bedding (1/4-in.; 7090, Harlan Teklad). When the rederivation program was initiated, conventional mice were housed in static autoclaved (sterilized) microisolation caging with standard irradiated diet (rodent diet 7912, Harlan Teklad), autoclaved municipal water in bottles, and autoclaved corncob bedding. Cages were changed on an open bench, and dirty cages were disassembled and stacked into component parts in the room.

Before and during the rederivation program, barrier mice were housed in static autoclaved microisolation caging with irradiated diet, autoclaved municipal water in bottles, and autoclaved corncob bedding. The cages were changed in hoods (Class II Type A biosafety cabinets, animal transfer stations, or

laminar-flow work benches), and dirty cages were disassembled and stacked into component parts in the room.

The mice used as surrogates mothers for cross-foster rederivation were housed in a separate limited-access room in static autoclaved microisolation caging with irradiated diet, autoclaved municipal water in bottles, and autoclaved corncob bedding. The cages were changed in a Class II Type A biosafety cabinet (model B40-ATS, Baker, Sanford, ME), and dirty cage component parts were reassembled in the hood before transfer to the cagewash area. Dedicated staff managed and performed all procedures in this room. Animal care staff were dedicated to conventional or barrier mouse rooms on a given day. Autoclaved nesting material (Nestlet, Ancare, Bellmore, NY) was routinely provided only for surrogate mothers. Two months after initiation of the rederivation program, the irradiated diet was changed to a nonirradiated rodent diet that contained fenbendazole (TD 01432, Harlan Teklad) for all surrogate mothers that received conventional mouse litters. Fenbendazole was not provided to the conventional mice as a cost-saving measure and to decrease the likelihood of antimicrobial resistance. All cages were changed in accordance with standard operating procedures for handling static microisolation caging and include the use of 100-ppm chlorine dioxide (MB10, Quip Laboratory, Wilmington, DE) as a disinfectant. Cages were sanitized in a cage-washer prior to autoclaving.

Determination of prevalence. Conventional mice were seropositive to MNV and MHV via multiplexed fluorometric immunoassay (MFIA), genus-specific fecal PCR-positive to *Helicobacter* spp.,[11,29] and positive for *Syphacia obvelata* by either perianal tape test, fecal flotation, or histopathologic findings (Table 1). Barrier mice were seropositive to MNV via MFIA and generic fecal PCR-positive to *Helicobacter* spp. (Table 2). Prevalence rates for MNV, *Helicobacter* spp., and MHV were determined by randomly sampling 18 cages in each mouse room (each mouse room had about 300 cages), averaging the mean values, and determining the standard deviation of the entire group. *Helicobacter* spp. prevalence in the conventional rooms was estimated to be same as that in the barrier rooms. Pinworm prevalence was estimated based on historic tests (tape test, fecal flotation, and histopatholgic data over a 15-y period). All the mice were free of mouse parvovirus 1 and 2, Theiler disease virus, rotavirus, Sendai virus, pneumonia virus of mice, reovirus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, *Ectromelia* virus, K virus, polyoma virus, mouse thymic virus, mouse cytomegalovirus, Hantaan virus, *Encephalitoozoon cuniculi*, and cilia-associated bacillus.

Animals. Surrogate mothers and males used to breed surrogate mice were either Hsd:ICR (Harlan Teklad) or Crl:CD1 (Charles River, Portage, MI). These vendors were free of mouse parvovirus 1 and 2, Theiler disease virus, rotavirus, Sendai virus, pneumonia virus of mice, reovirus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, *Ectromelia* virus, K virus, polyoma virus, mouse thymic virus, mouse cytomegalovirus, Hantaan virus, *Encephalitoozoon cuniculi*, and cilia-associated bacillus. Before pairing them to breed, mice were acclimated for 3 d after arrival. The mice that were rederived had various genetic backgrounds and specific gene additions or deletions. The procedures described in this report were approved by the University of Illinois at Chicago Animal Care Committee.

Cross-fostering procedure. The cross-foster procedure required 2 people. The first person examined pregnant donor mothers at least once every other day. Newborn litters were taken from their mother's cage, transported between 2 molded disposable face masks (Maytex, Shaoxing, China), gross contamination was removed, and given to a second person. The second person took the litter to the surrogate mouse room, where the litter was sprayed with 100-ppm chlorine dioxide and left wet for 2 min. The chlorine dioxide was blotted from the litter using a paper towel. The litter then was placed within the surrogate mother's nest after removal of the surrogate mother's litter. The surrogate mother's pups were euthanized by exposure to CO_2 followed by cervical dislocation. For the purpose of transferring scent to the donor pups, 2 of the surrogate mother's pups were left with their mother if they had a different coat color than the donor litter. Approximately 10% of the cross-foster litters died within the first week of birth. No litters were lost after the first week postpartum.

Sample collection. Surrogate mothers were euthanized at the time of weaning (4 wk). The mother was anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine (both given intraperitoneally), a blood sample was collected in a biological safety cabinet by means of cardiocentesis, and the mother was cervically dislocated. Serum samples were diluted 1:5 in PBS (pH 7.2; Sigma–Aldrich, St Louis, MO), frozen at –20 °C, and shipped within the week to the laboratory performing the tests.

Fecal samples were collected inside a biological safety cabinet. Between cages, forceps were sterilized at 121 °C for 17 s in a glass-bead sterilizer (Steri 350, Inotech, Rockville, MD). Fecal samples were put in 0.5-ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) and placed in sealed plastic bags. The samples were refrigerated at 5 °C after collection and sent to an overnight carrier by means of a courier without refrigeration within 1 wk of collection.[3]

Rederived mice were tested by MFIA for MNV (Charles River Labs, Wilmington, MA) by using surrogate mother serology at 4 wk after cross-fostering and viral nucleic acid fecal PCR of litters at 12 wk (Research Animal Diagnostic Laboratory, University of Missouri, Columbia, MO). Mice were tested for the presence of *Helicobacter* spp. using 16S ribosome nucleic acid (Research Animal Diagnostic Laboratory) at 4, 8, and 12 wk. Mice were tested via MFIA for MHV (Charles River) by using surrogate mother serology at 4 wk. Finally, mice were tested in-house for pinworms by fecal flotation and perianal tape test at 4 and 12 wk.

Positive litters were removed from the room immediately and not further tested. After identification of an MHV-positive cage, we performed serologic testing on 1 immunocompetent mouse per cage on the rack in which the positive cage was detected. Cages were tested every 2 wk for a minimum of 2 tests. We did not do follow-up testing when cages positive for *Helicobacter* spp. or MNV were identified.

Paradigms. Cross-foster rederivation followed 1 of 2 paradigms. Paradigm 1 included litters that were younger than 48 h at the time of cross-fostering. In this paradigm, the mouse litter may have been exposed to contaminated bedding that had been in the cage for up to 7 days.

Paradigm 2 included litters that were younger than 24 h at the time of cross-fostering. In this paradigm, the cage of the donor mother was changed daily after she was considered to be in late gestation. As a result, the mouse litter was exposed to contaminated bedding that had been in the cage for less than 24 h.

The litters were not selected prospectively into the different paradigms but were assigned the paradigm after a litter was born. Mice typically were checked daily or at least every 48 h. The mice from which litters were collected were adults younger than 1 y and were of the genotype identified by the investigator.

Table 1. Prevalence and Incidence of MNV, *Helicobacter* spp., MHV, and *Syphacia obvelata* in conventional mouse rooms

	Mean (range) of prevalence in conventional colony	Paradigm 1 Incidence			Paradigm 2 Incidence		
		Total no. of litters	No. of positive litters	% positive litters	Total no. of litters	No. of positive litters	% positive litters
MNV	85% (73% to 100%)[a]	57	3 (2 at 4 wk; 1 at 12 wk)[d]	5%	142	1 at 4 wk	0.7%
Helicobacter spp.	90%[b]	57	6 (4 at 4 wk; 2 at 8 wk)	11%	142	1 at 4 wk	0.7%
MHV	98% (88% to 100%)	57	7 at 4 wk	12%	142	0	0%
Syphacia obvelata	90%[c]	2[e]	2 at 4 wk	100%	1[e]	1 at 4 wk	100%

[a]The range is 2 standard deviations around the average prevalence of the rooms.
[b]Estimate of *Helicobacter* prevalence in the conventional mice based on our obtained value in the barrier rooms.
[c]Estimate of the *Syphacia obvelata* prevalence in the conventional mice estimated based on historic tape test, fecal flotation, and histopatholgic data
[d]Indicates the number of weeks after cross-fostering.
[e]None of the surrogate mothers were fed a diet containing fenbendazole. Pinworm treatment was instituted after the first 3 cross-fostered litters. None of these litters were included the total 287 conventional and barrier litters reported for the other agents.

Table 2. Prevalence and Incidence of MNV and *Helicobacter* in barrier mouse rooms

	Mean (range) of prevalence in barrier rooms	Paradigm 2 Incidence			Paradigm 2 Incidence		
		Total no. of litters	No. of positive litters	% positive litters	Total no. of litters	No. of positive litters	% positive litters
MNV	85% (49% to 100%)[a]	29	1 at 4 wk[b]	3.4%	59	0	0%
Helicobacter spp.	89% (58% to 100%)	29	5 (4 at 4 wk; 1 at 8 wk)	17%	59	1 at 4 wk	1.7%

[a]The range is 2 standard deviations around the average prevalence of the rooms.
[b]Indicates the number of weeks after cross-fostering.

Follow-up testing. The testing program for mice after the rederivation was as follows. Thirty-six tests are performed per testing interval in a 3000 mouse box facility. Each sentinel cage is used to test between 30 to 130 cages and is exposed to dirty bedding from these cages. One teaspoon of soiled bedding from each dirty cage is placed into the sentinel cage, mixed thoroughly, and a portion of this is used as bedding for the sentinel cage. Testing comprised: monthly serologic testing for MHV and MNV; quarterly fecal PCR *Helicobacter* spp. and MNV testing; quarterly perianal tape test and fecal flotation for pinworms; quarterly MFIA (Charles River Labs) for mouse parvovirus 1 and 2, minute virus of mice, MHV, rotavirus, Theiler disease virus, Sendai virus, pneumonia virus of mice, reovirus, and *M. pulmonis*; and annual MFIA for mouse parvovirus 1 and 2, Theiler disease virus, rotavirus, Sendai virus, pneumonia virus of mice, reovirus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, *Ectromelia* virus, K virus, polyoma virus, mouse thymic virus, mouse cytomegalovirus, Hantaan virus, *Encephalitoozoon cuniculi*, and cilia-associated bacillus.

Statistical methods. Statistical comparisons were made between the success rates of the 2 paradigms for all agents studied and between the success rates for mice from barrier and conventional rooms. The Fisher Exact Test (version 9.1, SAS Software, Cary, NC) was used for all comparisons. A P value less than 0.05 was considered as a significant result.

Results

A total of 287 litters (199 from conventional rooms and 88 from barrier rooms) were studied. Data from the first 3 conventional litters was not included in the 287 litters. Cross-fostered pups from these 3 litters were positive for *Syphacia obvelata*. This result prompted the change to a nonirradiated rodent diet with fenbendazole for surrogate mothers when conventional litters were cross-fostered onto them. After that change was made, no litters became positive for pinworms. Results from the conventional mouse rooms are presented in Table 1. Of the 199 litters cross-fostered, 3 were positive for MNV based on surrogate mother serology at week 4, and 1 was positive for MNV based on litter fecal PCR at week 12. The litter that tested positive by fecal PCR at week 12 was negative for MNV based on surrogate mother serology at week 4. Seven of the 199 cross-fostered conventional litters were positive for *Helicobacter* spp. based on litter fecal PCR at either week 4 or 8. Two litters of mice that were negative for *Helicobacter* spp. at week 4 became positive at week 8. Seven of the 199 conventional litters cross-fostered from rooms enzootic with MHV were positive for MHV based on surrogate mother serology.

Results from the 88 barrier-born litters are presented in Table 2. One of these 88 litters was positive for MNV based on surrogate mother serology at week 4, and 6 were positive for *Helicobacter* spp. based on litter fecal PCR at either week 4 or 8. One litter that was negative for *Helicobacter* spp. at week 4 became positive at week 8.

The success of eliminating MNV and *Helicobacter* spp. did not differ between conventional and barrier mice. Litters cross-fostered under paradigm 2 were significantly less likely to test positive for MHV ($P = 0.0002$) or *Helicobacter* spp. ($P < 0.0001$) than were litters cross-fostered under paradigm 1. Comparing

the 2 paradigms regarding the success in eliminating MNV yielded a *P* value of 0.08.

We had no evidence of horizontal disease transmission for any of the agents we were attempting to eliminate in the room that housed the surrogate mothers. Two years have passed since the last litter was rederived in this report, and we have had no evidence of contamination with these agents.

Discussion

Cross-foster rederivation allowed successful rederivation of mice contaminated with MNV, MHV, and *Helicobacter* spp. Using the procedures outlined here, we successfully used cross-fostering to rederive 262 of 287 litters (barrier and conventional). We initially used embryo transfer and late-term hysterectomy as methods of rederivation. However, cross-foster rederivation offered many advantages for our situation. For example, we needed a method that allowed rederivation of a large number of strains in a relatively short period of time. Minimizing the time that investigators had both clean and contaminated mice on campus decreased the risk of contamination of successfully rederived mice. In addition, cross-foster rederivation provided more flexibility because more personnel could be trained in the procedure. Unlike embryo transfer and hysterectomy, cross-foster rederivation did not require the euthanasia of valuable breeding females. Finally, depopulation[18] was not an option in our situation.

Biosecurity in the surrogate room was critical for success because more litters were likely to test positive during cross-foster rederivation than with embryo transfer and hysterectomy rederivation. The reassembly of dirty cage component parts inside the hood prevented aerosolization and subsequent contamination of the room that would have occurred had dirty cages been disassembled and stacked. Dedicated and trained staff were used to ensure that standard operating procedures were followed. Biosecurity of the surrogate mouse room was considered excellent in light of the lack of evidence of cross contamination between cages when a litter in the room tested positive for target agent(s).

Our strategy for biosecurity was early detection and elimination. Therefore, positive litters were removed from the room immediately. In addition, the room, racks, and cage exteriors were sanitized 3 times at 2-wk intervals after an agent was detected in the room. Chlorine dioxide was used to disinfect neonatal litters because it was readily available in the facility and known to be effective against *Helicobacter* spp., MNV, and MHV according to California Department of Pesticide Regulation. Iodine-containing disinfectant has also been used during cross-foster rederivation.[35] Changing cages daily reduces the exposure of the litters before cross-fostering.[9] In paradigm 2, the bedding and cages were changed daily during late gestation to increase the likelihood of success, and the data support daily cage changing as an effective means to prevent MNV and *Helicobacter* contamination. Murine hepatitis virus is a relatively fragile virus, and changing cages daily may not have made a great difference.

The selection of testing time points was based on obtaining multiple test results to increase the chance of identifying a positive litter within a 14-wk period. The 4-wk postpartum time point was selected because it coincided with weaning and was an early and convenient time to test the surrogate mother. The 8- and 12-wk time points were selected as intervals that allowed us to obtain results from more mature mice. Mice were not tested beyond 12 wk because of investigator need to use the rederived mice.

Murine norovirus does not appear to be transferred readily to neonatal litters.[8] However, 4 of the 287 cross-fostered litters had surrogate mothers that seroconverted to MNV at 4 wk, and 1 litter that was negative at 4 wk was positive by viral fecal PCR at 12 wk after cross-fostering. MNV contamination between paradigms 1 and 2 was not significantly different, although paradigm 2 tended ($P = 0.08$) to be less likely to yield positive test results. Perhaps a larger sample size would have shown a significant difference. The high prevalence of MNV in barrier rooms is consistent with previous reports[16] and likely is due to persistent viral shedding[15] and possibly environmental contamination.[7] Because MNV is environmentally stable, an important consideration regarding positive surrogate mothers was failure to decontaminate virus from the skin of the pups. Surrogate mothers may have become infected while grooming the pups. The positive PCR test at 12 wk is more difficult to explain. In this case, the negative MNV test at 4 wk could have been a false negative, or the surrogate mother may have seroconverted later. Mice that shed MNV typically seroconvert.[27] Cross-contamination from other cages was highly unlikely because no other contaminated mice were present in the room during the time this litter was rederived.

Thirteen of the 287 cross-fostered (barrier and conventional) litters were positive for *Helicobacter* spp.; 10 were positive at week 4, and 3 were positive at week 8. Therefore, we recommend that litters be tested for *Helicobacter* spp. at 8 wk after cross-foster rederivation before release to the animal holding area. Our findings are consistent with other reports on the effective use of cross-foster rederivation in elimination of *Helicobacter* spp.[10,12,30,31,35] and MHV.[22,35] More specifically, our data support a previous recommendation[30] that litters younger than 24 h be used when trying to prevent the horizontal transmission of *Helicobacter* spp. from the mother to her newborn litter. Litters of paradigm 2, which were younger than 24 h, were significantly less likely to test positive for *Helicobacter* spp. and MHV after cross-foster rederivation.

Some scientists[36] have raised concerns about in utero transmission of *Helicobacter hepaticus* in immunodeficient mice because it has been isolated from late-gestation SCID mouse fetuses.[21] The majority of mouse strains rederived at our facility were characterized as being immunocompetent. However several genes were targeted (knocked out) in various mouse strains to render the mice deficient in various aspects of the immune system (Table 3). Only 1 B6.129 × 1-*Mpo*^{tm1Lus} litter from these strains tested positive for *Helicobacter* spp. None of these strains tested positive for MNV or MHV after cross-foster rederivation.

Adjunct methods for preventing *Helicobacter* spp. contamination of newborn litters include removing the male from the cage prior to parturition[9] or feeding a diet containing antibiotics[12,17,19,20] to the surrogate mother. Feeding medicated food could be used if litters are not robust at birth and an extra day on the natural mother would be considered beneficial or if a surrogate mother is not available. Triple- and quadruple-antibiotic diets (Bioserve, Frenchtown, NJ) are available for this purpose. These diets are relatively expensive and not entirely reliable.[12,36] Removing the male prior to parturition extends the cross-fostering period needed to yield *Helicobacter*-free mice.[9]

Murine hepatitis virus is highly contagious and was readily detected at 4 wk by surrogate mother serology. Our data support a previous report[22] in that we found no MHV-positive litters when litters were cross-fostered at 24 h or younger. Our data differ from another previous report,[35] in that we found MHV-positive litters when they were cross-fostered at 48 h or

Table 3. Genes of rederived mice targeted to render mice immunoincompetent

STOCK-Ifnartm1Agt	STOCK-Ifngtm1Ts	B6.127S7-Fah/\ exon5,Rag1tm1Mom/J	B6.129X1-Mpotm1Lus
B6.129-Pik3cgtm* (University of Connecticut)	B6;129- Rac2tm1Din	B6.129-Sphk1tm1Rlp	B6.129-Sphk1tm1Rlp
B6;129-Myd88tm1Mas	B6;129-Inpp5dtm1Pngr	B6.129-Inppl1tm* (IRIBHN, Brussels, Belgium)	C.Cg-Ifnartm* (Zurich, Switzerland)
B6.129-PrkccNaka	B6;129-Dusp10tm1Cdo	STOCK-Rgs2tm1Phgr	B6.129-Rorctm1Drl
B6.129X1-Prkcqtm1Drl	B6;129-Ppp3catm1Jmol	B6.129-Sod3tm1Mrkl	

younger. However, these data are difficult to compare because in our case, the prevalence of MHV likely was decreasing with time once use of microisolation filter tops was initiated. Most of the MHV-positive litters occurred early during the process. In contrast, the prevalence of *Helicobacter* spp. and MNV in the barrier mice likely did not decrease during this process.

Only when combined with feeding surrogate mothers fenbendazole-containing diet did cross-foster rederivation eliminate *Syphacia obvelata*. This report is the first in which fenbendazole-containing diet has been used in conjunction with cross-foster rederivation to eliminate *Syphacia obvelata*.

In conclusion, we have demonstrated that a cross-foster rederivation technique in combination with intensive testing and removal of positive litters after cross-foster can successfully eliminate MNV, *Helicobacter* spp., and MHV from a contaminated colony. We also have shown that 12 wk of age in the case of MNV, 8 wk of age in the case of *Helicobacter* spp., and 4 wk of age in the case of MHV are reliable time points when testing for these agents. Two years have passed since the last litter in this report was rederived, and we have had no evidence of contamination with the eliminated agents.

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