A Dissertation for the Degree of Doctor of Philosophy

Quality control for specific pathogen free mouse facility using embryo transfer and exhaust air dust PCR

수정란 이식과 배기 공기 분진 중합효소 연쇄반응을 이용한 특정 병원체 부재 마우스 시설의 정도 관리

By

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February 2023

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(Major: Laboratory Animal Medicine)
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A dissertation submitted to the Graduate School in
Partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

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December 2022

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ABSTRACT

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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, I 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.

Health monitoring is essential for ensuring animal health and reliable research results. Each animal facility should establish adequate health monitoring methods, and microbiological quality control should be implemented through regular health surveillance. Recently, specific pathogen free (SPF) mice have been housed in individually ventilated cage (IVC) racks in the majority of mouse facilities globally, and health monitoring is implemented using a soiled bedding sentinel (SBS). Even though SBS monitoring is a standard method, it has a limitation in that some pathogens are not sufficiently transmitted to the sentinel housed in the IVC. The exhaust air dust polymerase chain reaction (EAD PCR) method has been reported to be a reliable complementary method to SBS monitoring based on research findings. In Korea, health monitoring programs using EAD PCR have not yet been applied to laboratory animal facilities. The microbiological status of mouse

colonies housed in the two IVC racks was compared using SBS and EAD PCR monitoring in our SPF mouse facility. Except for *Helicobacter* spp. and *Staphylococcus aureus*, the detection of 16 pathogens did not differ between the two methods. In the detection of *Helicobacter* spp., EAD PCR was found to be more sensitive than SBS. *Helicobacter* spp. were not found by SBS, whereas four *S. aureus* positive samples were detected by either SBS or EAD PCR test. According to our findings, EAD PCR can be used as a supplement to SBS monitoring. Moreover, EAD PCR can reduce the number of animals used, making it a 3R (Replacement, Reduction, Refinement)-consistent method.

In conclusion, the elimination of infectious pathogens using embryo transfer and regular microbiological monitoring using SBS and EAD PCR are crucial for the quality control of the SPF mouse facility. The reliability of animal test results and 3R compliance can be assured through quality control of SPF mouse facilities.

Keywords: SPF mouse, Pathogen, Embryo transfer, Rederivation, Soiled bedding sentinel (SBS), Exhaust Air Dust (EAD) PCR

Student number: 2016-30496

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LIST OF ABBREVIATION

SPF	Specific pathogen free
MNV	Murine noro virus
IBD	Inflammatory bowel disease
Tego	Alkyl diamino ethyl glycine HCl 5%
GEM	Genetically engineered mouse
PMSG	Pregnant mare serum gonadotropin
Hcg	Human chorionic gonadotropin
IVC	Individually ventilated cage
SBS	Soiled bedding sentinel
EAD	Exhaust air dust
PCR	Polymerase chain reaction
RT PCR	Real time polymerase chain reaction
ELISA	Enzyme linked immunosorbent assay
3R	Replacement, reduction, refinement
LDEV	Lactate dehydrogenase elevating virus
RO	Reverse osmosis
IVF	In vitro fertilization
ICSI	Intra cytoplasmic sperm injection
MVCS	Micro ventilation cage system
ACU	Air control unit

HEPA	High efficiency particulate air filter
FELASA	Federation of European laboratory Animal Science Association
MHV	Mouse hepatitis virus
MVM	Minute virus of mouse
MPV	Mouse parvo virus
PVM	Pneumonia virus of mice
EDIM	Epizootic diarrhea of infant mice
TMEV	Theiler's murine encephalomyelitis virus
SCID	Severe combined immunodeficiency
NOD	Non obese diabetic
NSG	NOD SCID gamma
OB	Opportunistic bacteria
IL10	Interlukin10
ICLAS	International Council for Laboratory Animal Science

LITERATURE REVIEW

1. Microbiological quality of animal

The validity and consistency of research findings as well as animal welfare can be significantly impacted by the microbiological quality of laboratory animals. For breeding and laboratory facilities, it is important to set up a laboratory animal health surveillance program as a vital part of all quality assurance procedures. All breeders and users of laboratory animals including mouse, rat, Syrian hamster, guinea pig, rabbit, and diagnostic laboratories are advised to follow these suggestions. They go through important HM components such medication selection, tissue and animal choice for testing, sampling frequency, basic test methods, result interpretation, and HM documentation. The use of surveillance animals, opportunistic pathogens, and the principles of HM are all given more importance in this recommendation than in previous ones (particularly under cage-level isolation conditions). (1)

2. Microbiological unit

Microbiological "units" are used to structure and organize animal facilities. An independent microbiological object having distinct areas and traffic for people, animals, and materials is referred to as a "microbial unit." Units and HM plans specific to their application are defined by the person responsible for designing the HM program. Therefore, various monitoring programs may be needed for the same institution. (1)

A crucial element in the design of HM programs is the determination of microbiological units since it affects the sampling strategy, the type and frequency of testing, and the evaluation of the findings. In fact, risk factors of microbial contamination and results can differ between breeding and experimental units, and HM program design should take these differences into account. (1)

Cage-unit isolation housing, like individually ventilated cages (IVCs), is increasingly often used in laboratory mouse facilities. Although it relies on housing and handling practices, the transmission of fomites or other infectious agents originating from animals between cages is typically minimized when compared to open cages. IVC housing may limit the spread of allergens and pathogens. Under these circumstances, HM becomes a challenging task, necessitating the development of strategies for proper sampling, irrespective of the statistical aspects mentioned in earlier guidelines, for breeding and experimental groups. (1)

Table 1 Microbiological unit instances (1)

Microbiological unit instances
Barrier facilities (including one or more rooms) where animals are kept in open cages or where staff, equipment, and animals can move freely.
An isolator
A set of micro isolation cages where animals can directly interact and spread the infection horizontally. A solitary cage (individually ventilated cage operated in a laminar flow workstation with stringent sanitary criteria)

3. Factors for determination of pathogen introduction to facility

The completeness of health monitoring program should consider the degree of risk induced on a specific unit as well as the level of risk that unit causes to other units (Table 2). Both biological features and the prevalence of infectious microbes influence how frequently objects are monitored. Pathogens that are highly infectious and prevalent should be screened often. The frequency of monitoring may also be a reflection of the agent's potential influence on ongoing research projects. (1)

New pathogens and HM methods are also taken into account. The HM program's purposes remain the same, only the methods of reporting or monitoring are altered by new pathogens and procedures. The presence of a pathogen not included in these recommendations but suspected to be important should be reported in follow up documents and controlled equally as pathogens listed. (1)

The HM program need to make it possible to evaluate the protective measures installed on the equipment with accuracy. The program design should take into account the animal species, immunological condition, microbiological unit and number of animals per unit, as well as the testing frequency, the animals and organisms from which samples are taken, the organisms to be tested, detection techniques, and equipment. Selecting each animal species independently in microbiological units with multiple animal species should be done. Moreover, with diverse ages and genetic backgrounds, susceptibility and the serological response to infection vary. The animals chosen for monitoring should be of different ages. Instead, the sampling should alternate over period between populations or strains. (1)

Table 2. Some factors that determine the risk of introducing unwanted agents into an animal unit. (1)

Higher risk
Animals are introduced on a regular basis (at least once per month).
Closely spaced units with different microbiological state.
Animals from various breeding colonies are introduced (from multiple breeders).
Animals are moved out of the unit for manipulation, then reintroduced.
Insects and wild or uncontrolled rodents having access to animal rooms or feed and bedding depots.
The unit is frequently exposed to biological materials derived from the same species of animals.
Facilities for a variety of experiments.
The facility is frequently accessed by research staff.
The unit's animal care staff rotate often.
Equipment that is shared and cannot be easily sterilized (behavior, imaging).
Lower risk
Closed animal colony for breeding.
'All in–all out' system.
Periodical introduction of animals.
Limited types of experiments.

4. Health Monitoring

The fitness of animals for use in research can be affected by environmental and genetic factors and how they interact (2) (3) source of infection development. The animal welfare, experimental variation, and success of scientific research activities are directly impacted by the microbiological quality of animals in confined or laboratory animal facilities, highlighting the significance of this factor. (1) To ensure that experimental results can be replicated, it is crucial to use animals with recognized biological characteristics. These suggestions' main goals are to increase understanding of the microbiological standards of research animals and to assist in meeting ethical, moral, and legal standards for health monitoring (HM) programs including design, sample collection, surveillance, documentation and interpretation.

Most infections don't result in obvious clinical symptoms. The absence of illness symptoms has therefore merely minor diagnostic relevance. On the other hand, animal research outcomes may be significantly impacted by latent or subclinical infections. Laboratory animal physiology (behavior, growth rate, relative locomotion, body weight, immune response) (2) (4) use of animals has increased. Latent animal infection can result in contamination of tissues, cell lines, serum, or biological material such as embryos and mates (5) (6). Such contamination may stop the item from being used or may infect new animals. There is some evidence that some viral illnesses that affect laboratory animals can potentially affect humans (7). Because of all of these factors, it's crucial that every institution develops a laboratory animal HM program as a vital component of any quality control process. Although the expense of HM and preventative procedures may appear costly, it is rather inexpensive compared to the overall cost of the research work. The Federation of

European Laboratory Animal Science Associations (FELASA) recommendations for the certification of HM programs and diagnostic labs can be used to accredit institutional HM programs and laboratories (8).

The FELASA HM Recommendations are designed for both commercial and non-commercial breeders, as well as users of laboratory animals including animal facility staff, veterinarians, scientists that utilize animals for research objectives, and testing laboratories). It establishes a structure for HM of laboratory animals in breeding and experimental populations, with the main goal of coordinating processes across nations involved in FELASA (1).

Table 3. Recommended infectious agents to monitor and frequencies of monitoring for laboratory mice (1)

	Every 3 months	Annually
Viruses		
Mouse hepatitis virus	x	
Mouse rotavirus	x	
Murine norovirus	x	
Parvoviruses:		
Minute virus of mice	x	
Mouse parvovirus	x	
Theiler's murine encephalomyelitis virus	x	
Lymphocytic choriomeningitis virus		x
Mouse adenovirus type 1 (FL)		x
Mouse adenovirus type 2 (K87)		x
Mousepox (ectromelia) virus		x
Pneumonia virus of mice		x
Reovirus type 3		x
Sendai virus		x
Bacteria		
Helicobacter spp.	x	
If positive, speciation for *H. hepaticus, H. bilis* and *H. typhlonius* is recommended		
Pasteurella pneumotropica	x	
Streptococci β-haemolytic (not group D)	x	
Streptococcus pneumoniae	x	
Citrobacter rodentium		x
Clostridium piliforme		x
Corynebacterium kutscheri		x
Mycoplasma pulmonis		x
Salmonella spp.		x
Streptobacillus moniliformis		x
Parasites		
Endo- and ectoparasites (reported to the genus level)	x	
Additional agents*		
Viruses:		
Hantaviruses		
Herpesviruses (mouse cytomegalovirus, mouse thymic virus)		
Lactate-dehydrogenase elevating virus		
Polyomaviruses (mouse polyomavirus, K virus)		
Bacteria and fungi:		
Cilia-associated respiratory bacillus		
Klebsiella oxytoca, Klebsiella pneumoniae		
Other *Pasteurellaceae*†		
Pneumocystis murina		
Pseudomonas aeruginosa		
Staphylococcus aureus		
Others as necessary		

Regardless of when they were detected, all pathogens on the list should be recorded when they are found in a diagnostic test. *It is optional to test for these infections

however it is recommended if there is a special need. Testing frequency is determined by regional factors. The inclusion of the *Pasteurellaceae* family is debatable, I am aware of that. Families can be chosen if facilities are needed, but this must take into account the fluidity of accurate phenotyping as well as the inaccuracy of some commercial kits when it comes to identifying *Pasteurella pneumotropica.*

5. Prevalence of infectious agents

Pathogens which are more common than those that are rare pose a greater danger of contamination. The development and use of detecting method, and preventative or therapeutic actions are all factors that affect prevalence in addition to the distinctive qualities of the animal or biological material. Selecting the pathogens to monitor can be supported by prevalence statistics (9) (10) (11) (12) (13) (14) (15). Animal strain, immunological state, age, and gender, as well as the local conditions of housing containment, like open cages or micro-isolated cages, can also be used to predict the regional prevalence of an infectious agent. It might be influenced by a variety of additional elements, including work patterns, the amount of animal transportation within and between facilities, within and between units, and among individual animals. (1)

6. Progress in Health Monitoring

Establishing a solid basis for maintaining animal health and guaranteeing the reliability of results used in biomedical research is health monitoring (HM) of experimental rodents. To enhance the microbiological quality of mice housed in laboratory research institutions, significant advancements have been made in animal housing and gnotobiotic rederivation procedures throughout the past century. The hygienic condition of animals used for research has improved over the past 140 years, from the early domestication stages (1880–1950), when breeding colonies were developed with animals naturally infected with different pathogenic microbes, to a time when prevalent viruses and different specific agents were eradicated from laboratory rodent population (1980-1996).These broadly segmented period of time, first detailed in the late twentieth century by Steven H. Weisbroth and David G. Baker(16), may be extended to the most recent and still continuing scenario, the stage of separated animal husbandry with limited pathogen exposure. Despite the fact that this evolution follows an inherent logical route, it emerges out that the present state also presents its own set of obstacles (17).

The microbiological quality of laboratory animals has significantly improved because to the widespread application of sanitation practices and hygiene monitoring systems in recent years, creating breeding colonies free of pathogens as well as the majority of opportunistic agents(16). This enables effective management of the emergence of infectious diseases, providing rigorous barrier husbandry in commercial supplier and laboratory animal facilities. Recent rodent research management has definitely inhanced the microbiological quality of an entire animal colony, in keeping with the 3Rs(18), and made it possible to produce even critically

immunodeficient animal models without microbial-induced infections. Furthermore, because the roles of genes and noncoding genomic sections remain mostly undetermined, the massive and fast increasing supply of genetically engineered animals results in an uncertain number of mutants with immunological abnormalities, necessitating stringent barrier housing (17).

7. Scope of HM management

The variety of HM concepts expanded over time as the microbiological quality of laboratory animals improved (Fig. 1). Naturally infected animals for breeding could have carried a range of pathogens in the beginning stages of rodent maintenance, causing severe clinical disease and rapid loss of entire animal populations. The major goal at the time was just to keep animals alive. How can I later identify and use the causal agent? Common pathogens were eliminated from animal populations and redirected to questions using reintroduction processes such as hysterectomy or offspring embryo transfer of infected donor animals and raised with uninfected surrogate mothers. What can you do to keep them healthy? (16)

The scientific community has become increasingly aware that even asymptomatic infections with specific agents cause distinct consequences in various animal models, resulting in the development of HM, as a result of the development of accurate and more sensitive testing (screening) techniques. The emphasis is on elements of scientific validity (how can quality be guaranteed?). Recommendations for HM program (1) (19) (20) that identifies specific pathogens to be included in HM processes and aims for a high degree of hygiene consistency. Repeated reintroduction cycles enable certain rats to maintain artificially constrained microbial communities. In processes, these "inclusions" of microbial preparations frequently create the point of maximal "exclusion" in colonies (21) (22).

The major influence of microbial community composition on animal models is now becoming more widely recognized thanks to ongoing advances in diagnostic procedures and the quick development of molecular technologies (23) (24) . To ensure biomedical value (how can their validity be guaranteed?), modern

HM paradigms must take these consequences into account. Among other things, this necessitates the incorporation of metagenomic technologies and a distinct viewpoint on hygiene control (17).

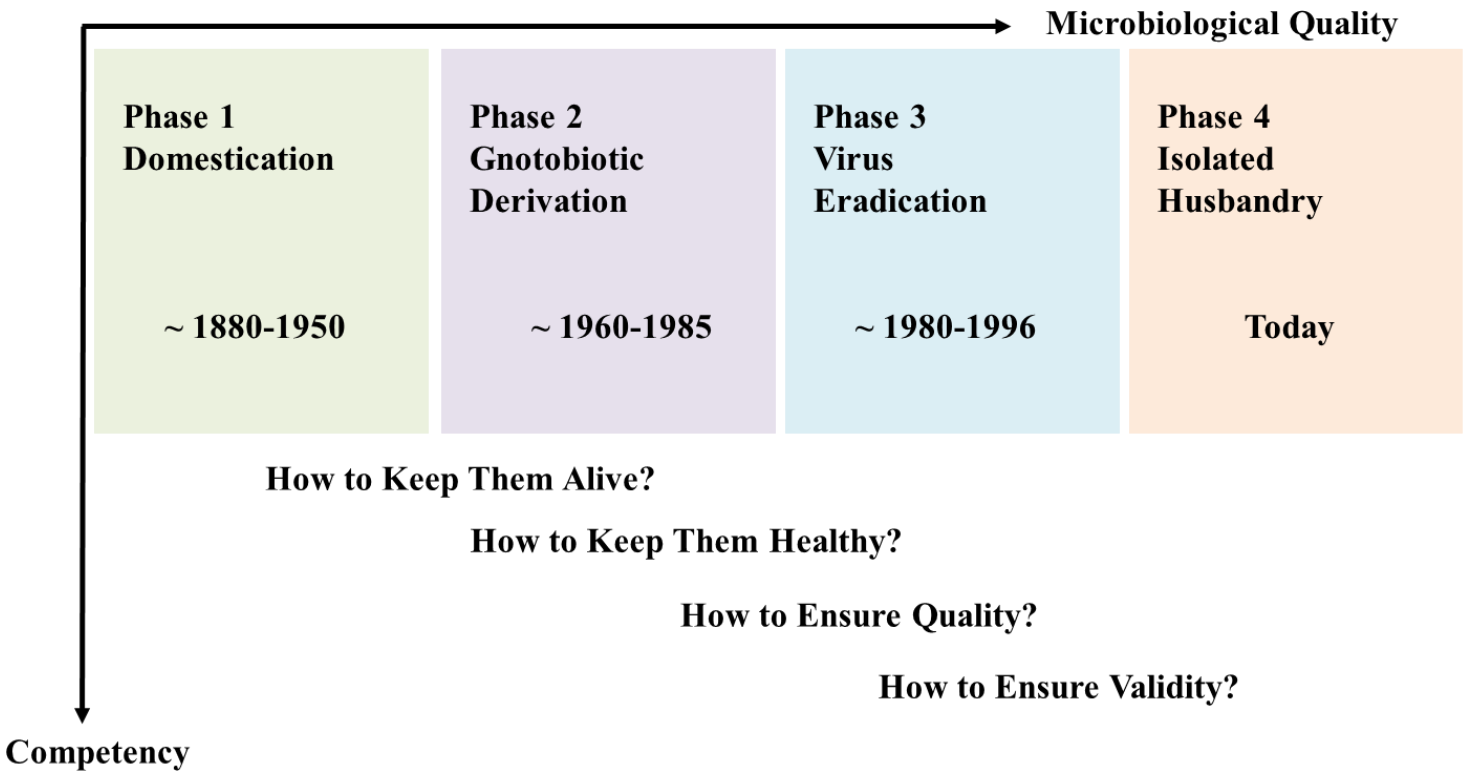

Figure 1. Schematic timeline roughly dividing the last 140 years of experimental rodent work into four different phases. (16) (18)
Both microbiological quality improvement in laboratory rodents and a wide variety of health monitoring concepts have developed. The underlying questions show the qualifications needed to these notions into practice or make them real.

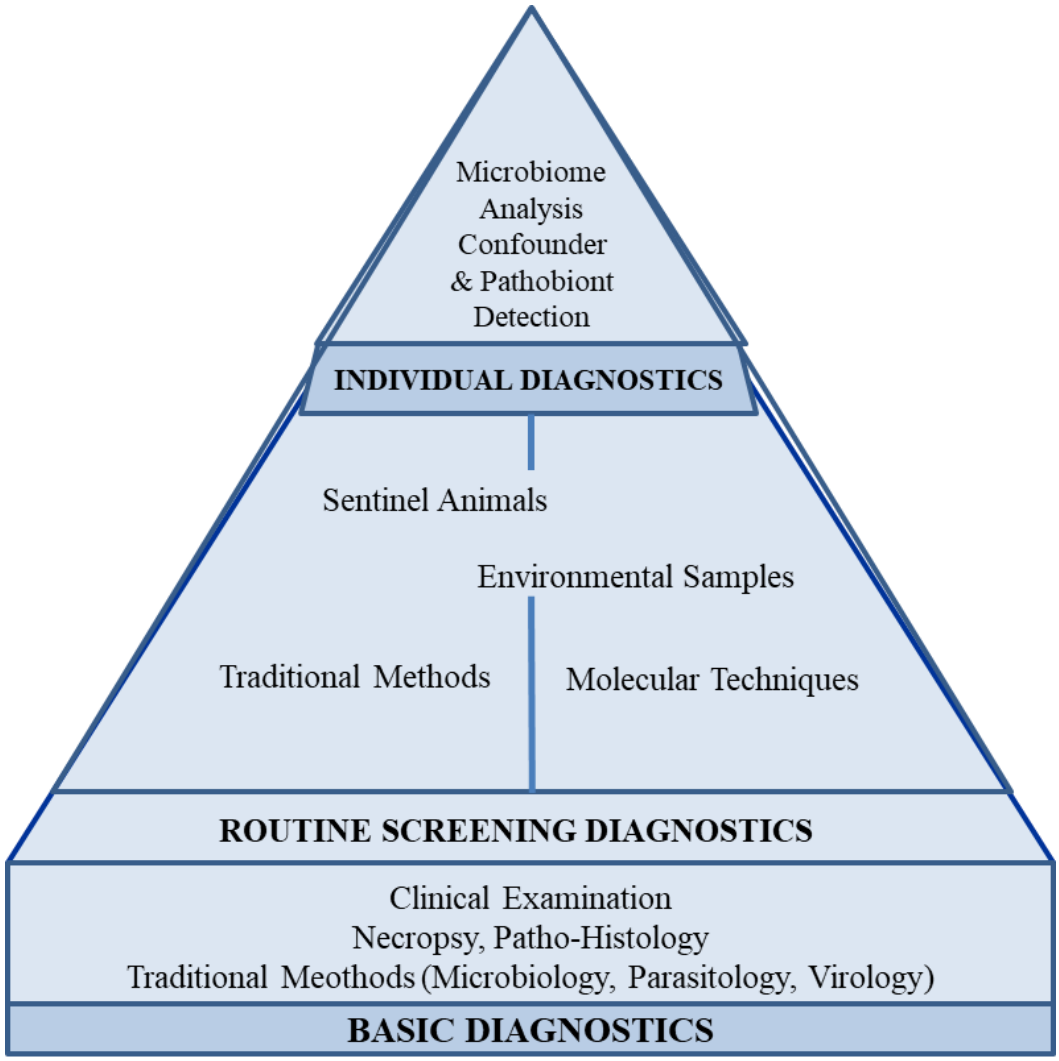

Figure 2 Diagram depicting the different proportions of HM programs (17) .
The primary body of HM concepts should be composed of regular diagnostic screening procedures, which should serve as a strong foundation, and specific diagnostic techniques as supplementation.

8. Pathogens Affecting the Animal's Health Status

Majority of infectious pathogens affect young animals most severely during the weaning stage, when additional stresses and the lack of maternal antibodies enable for unregulated pathogen proliferation (16) (25). Acquired diarrhea in infant mice (EDIM) and murine rotavirus type B (26) (27) (28) (29) (30) induced by mouse rotavirus type A as a result of rotavirus infection are typical examples of age-dependent illness progression. Although all animals are vulnerable, clinical illnesses including loose stools, lethargy, and abdominal distension only manifest in infected animals about two weeks after birth, which is why EDIM/IDIM is used to treat Tyzzer's disease associated with diarrhea or abdominal distension. It sets it apart from other infections like the enterotropic type of the MHV. (17)

These significant age-dependent varieties in virus susceptibility are significant benefits in aspects of hygienic practices because they allow for relatively easy virus elimination through extended breeding cessation and stringent sanitary standards, as was done following an EDIM outbreak in an experimental barrier housing mice (31). The real incidence of mouse and rat Rotaviruses is currently low due to sanitation protocols and stringent sanitary procedures; nonetheless, outbreaks sometimes occur, as was recently documented after exported mice were exposed to contaminated transport boxes (32).

In general, chronic respiratory disease is relatively susceptible to affect rodents. *Phyllobacterium rodentium*, formerly known as cilia-associated respiratory (CAR) Bacillus or *Mycoplasma pulmonis*, is extremely contagious in rats. Particularly if animals are susceptible to it by poor housing conditions or co-infection with other pathogens, this infection can result in serious respiratory tract infections.

pathogens (opportunistic) (33) (34) (35) (36) (37) (38) (39). The majority of viral infections have been eliminated from experimental mouse and rat populations, making opportunistic bacteria the most frequent danger to animal health. In this setting, the *Pasteurellaceae* lineage can produce inflammatory diseases including conjunctivitis, dacryodenitis, or numerous abscess forms in susceptible mouse and rat strains while frequently being a normal component of mucosal regions' normal flora. As a result, it can be categorized as a classical pathogen (40) (41) (42) (43) (44) (45). The rodent *Pasteurellaceae* was recently reclassified as a new genus, *Rodentibacter,* together with *Rodentibacter pneumotropicus* and *Rodentibacter. heylii* (formerly known as *Pasteurella pneumotropica*) is the most prevalent species in laboratory mouse and rat facilities (46). *Staphylococcus aureus* and *Klebsiella spp*. are common opportunistic pathogens in laboratory rodents, and both are zoonotic bacteria, therefore infections can be transmitted from animals to humans or vice versa. In two previous studies, however, host-adapted S. aureus strains were detected primarily in laboratory mouse and rat colonies responsive to methicillin treatment (47) (48). Even though both pathogens are not necessarily harmful to animals, although sensitive strains may develop clinical symptoms due to natural bacterial colonies.

C3H/HeJ mice, which express a malfunctioning Toll-like receptor 4 and have compromised innate immune responses due to poor pathogen recognition, developed otitis media brought on by *Klebsiella oxytoca* on numerous occasions (49) (50). Additionally, *Klebsiella* caused pneumonia and urogenital infections in LEW.1AR1iddm rats—animals predisposed to type 1 diabetes mellitus caused by autoreactive T-cell populations (49). Two independent studies described the

development of Staphylococcus aureus-induced Botryomycosis and facial abscesses in susceptible mouse outbred strains although disease development in these cases occurred in some way immune-modulated animals (51) (52).

Several animals, for instance, immunodeficient mouse and rat strains, develop severe intestinal inflammation when infected with Helicobacter spp (53) (54) (55) (56). As Mangerich et al. reported after infecting Rag2-deficient mice, particularly *Helicobacter hepaticus* can also cause other diseases like hepatitis and inflammation-induced colon carcinogenesis in this situation (57). Co-infections are common and may deteriorate development of the disease because at least nine different Helicobacter species (*H. hepaticus, H. typhlonius, H. bilis, H. rodentium, H. ganmani, H. muridarum, H. mastomyrinus, H. rappini, and H. trogontium*) have been identified from the digestive tract of laboratory rodents (58) (59) (60). Undefined intestinal inflammation may happen spontaneously after exposure to combined opportunistic agents because the majority of genetically modified immunodeficient mouse and rat strains do not have enough pathogen defense mechanisms. As a result, those animals are generally susceptible to developing infectious diseases (61). The same factors also contribute to the development of other diseases in immunocompromised animals following infection with microbes. In this regard, it was recently reported that NSG and NRG mice developed ascending pyelonephritis after surgery, which might be connected to a *Candida albicans* infection acquired following an intravenous injection inside a restraint device (62).

Numerous reports have been made of cases of otitis media caused by *Corynebacterium bovis* (63) (64) and *Ralstonia picketii* (65), as well as cases of otitis media developing as a result of severe sepsis(66) following bacterial translocation

of commensal bacteria in the normal intestinal flora. Pneumocystis sp. chronic wasting owing to infection is one of the biggest health risks to animals with compromised immune systems (67) (68). Even in immunocompetent animals, pulmonary inflammation can develop sometimes (69), but an adequate adaptive immune response typically prevents progression of disease. However, subclinically infected animals can transfer pathogens to susceptible hosts, causing severe clinical signs in immunocompromised animals of the colony (70), and co-infection with different opportunistic pathogens can aggravate the overall pathogenesis (71) (72).

9. Factors for Standard Health Monitoring

Test accuracy and sample size are often the two parameters that have the greatest impact on full diagnostic success (implicating the adequacy of the procedure selected). The sensitivity and specificity of diagnostic tests serve as indicators of their accuracy (Figure 3). The first is just somewhat significant for the likelihood of detecting a specific infection source, while the second establishes the diagnostic value of the findings. Greater diagnostic sensitivity diminishes the number of false positive cases and lowers the quantity of false-negative outcomes. With fewer false-positive findings, diagnostic specificity is increased, minimizing the number of false negative cases. Both parameters are crucial for understanding the findings since they define the test procedure's positive and negative predictive values. Statistics may be used to determine sample size, which establishes the minimum number of animals that must be screened in order to reliably identify pathogens within a colony. (17) The underlying equation (shown in Figure 3) is made up of the intended confidence interval (indicating the maximum risk of a false negative result), the test's sensitivity, the estimated prevalence of the pathogen being checked, and the size (number of animals) of the relevant population (1) (73) (74) (75).

It is crucial to remember that this equation only applies in situations when pathogens may randomly infect all animals within a herding group, which is highly improbable under the conditions of present Individually Ventilated Cage (IVC) confinement. As a result, alternatives to randomly selecting colony animals are needed for husbandry forms that confine animals to cage-level containments. These alternatives can be achieved by utilizing sentinel animals (17).

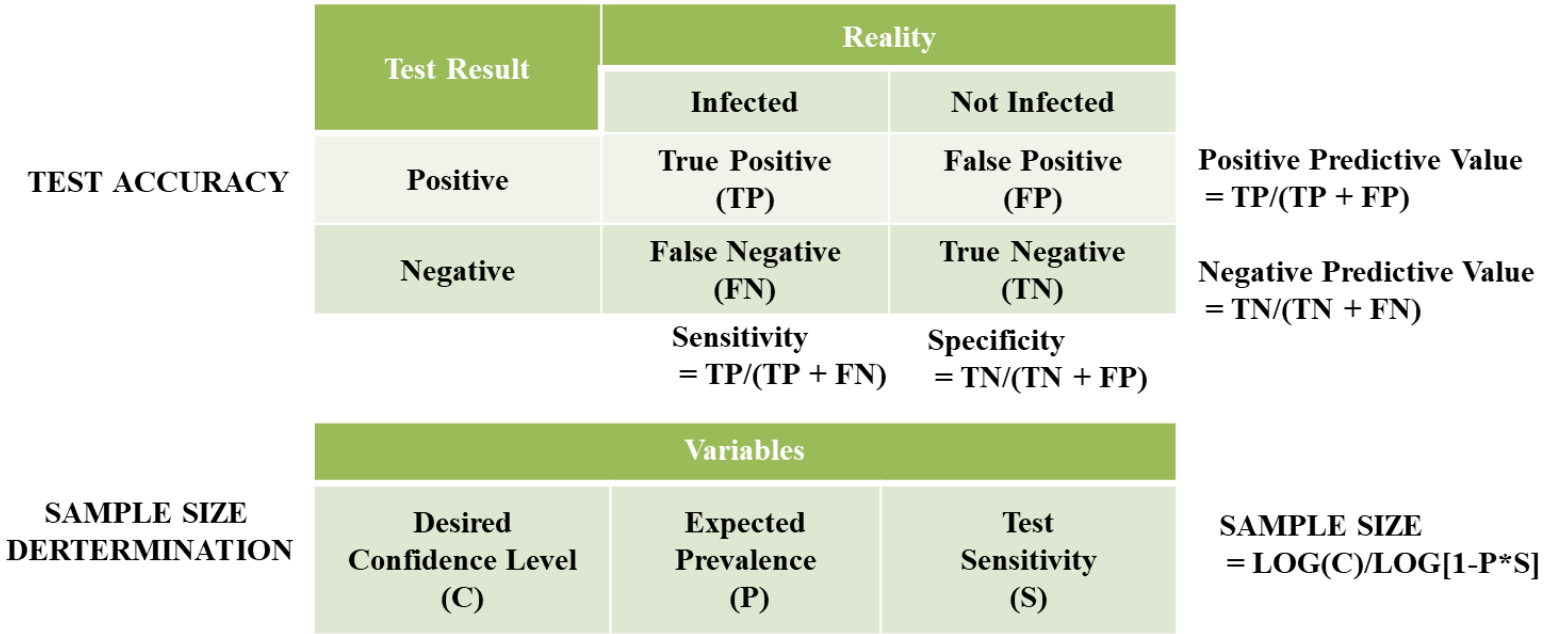

Figure 3. Schematic overview of the two main factors influencing the diagnostic success. (1) (73) (74) (75)

The diagnostic sensitivity and specificity of the test, together with the underlying positive and negative predictive values, all serve to explain the test accuracy. Both true positive (TP) and negative (TN) findings as well as false positive (FP) and negative outcomes can be used to calculate all parameters (FN). The desired confidence interval (C), anticipated pathogen prevalence (P), and the appropriate test sensitivity are used to determine the number of samples needed to identify an infectious agent (S). Importantly, this figure only applies to colonies with at least 100 animals and free distribution of infections.

10. Animals for testing and sampling

For reliable findings from the HM program, it is essential to carefully choose the animals and samples to test. The most accurate information on a unit's microbiological state comes from the animals that live there, hence wherever feasible, HM should be conducted on these animals. Animals from as many rooms as feasible should make up samples in microbiological units with several rooms. Because each IVC may indicate a separate microbiological component of the rack in which it is housed, it is crucial to highlight that as many cages as feasible should be tested. Animals that are sick, deceased, or have their samples taken from them must be sent for testing in addition to those that are scheduled for regular monitoring. This holds true not only for animals used in experiments but also for captive animals that show unexpected phenotypes. Researchers can distinguish between the effects of the experimental protocol and those of infection using necropsy. Results from necropsy may lead to increased sample size, monitoring frequency, or additional pathogen testing (1).

11. Sentinel

Sentinels are purposefully exposed to infectious substances with the goal of infecting them with the pathogens existing in a housing environment. Microbiological monitoring of sentinels can serve as a reliable indicator of the animal colony's health. Depending on the type of pathogen exposure, there are various sentinel system types. The most typical method is exposing sentinels to the dirty bedding of the resident animals (referred to as soiled bedding sentinels), which was reported as standard monitoring system for infectious pathogens that are transmitted into the animals' feces, urine, or other excretions (76) (77) (78) (79) (80) (81) (82).

It is possible to prevent unintended "dilution" of infectious agents and reduce the pathogen burden by putting sentinel animals directly in the colony's fully utilized cages, where they also come into touch with food, water, and nesting materials. Since it has been demonstrated that essential pathogens like *Rodentibacter spp.* were detected by this method, the diagnostic reliability will likely be higher even if this methodology necessitates a longer amount of time to monitor entire IVC rack systems (83). However, soiled bedding sentinels are not ideal for the accurate detection of ectoparasites (such as fur mites) or pathogens, which are only transferred by aerosols (such as SeV), irrespective of the concrete variant (78) (84) (85) (86). So different sentinels (direct contact sentinels or exhaust air dust sentinels), resident animal health monitoring, or environmental PCR analysis are required in those circumstances.

Some sentinels can be maintained in direct contact with the animals to be tested by housing them in the same cage because some pathogens such as LCMV,

Pasteurella pneumotropica, Sendai virus, cannot be easily transmitted through dirty bedding (87) (78) (88) or exhaust air (e.g., *Helicobacter spp*., mouse rotavirus, mouse parvovirus) (78). Direct contact sentinels may enable the detection of certain pathogens or at the very least raise the probability of their detection. Animals to be examined should be compatible with contact sentinels. Contact sentinels shouldn't be moved between cages as this could transfer an agent to cages that weren't previously infected (1) .

12. Recent Developments in Standard Health Monitoring

PCR based diagnostic technology technology has been thoroughly verified as a suitable alternative to standard culture, microscopy, and serological detection methods during the last few decades. PCR testing can identify extremely minute quantities of nucleic acids from varied samples and can detect the exact gene base sequence of the source of infection. Alternatives to direct animal testing include environmental sampling materials (89). Because infected animals release pathogens through several pathways (feces, urine, saliva, various excretions, skin/fur) in cage environment, this sample can be evaluated rather than screening specimens collected from the animals.

Relying on pathogens, numerous types of samples can be utilized at the single cage (bedding material, nesting stuff, dried feces, cage swabs, or cage lid filter material) or rack level. An efficient option, especially for screening larger animal populations, might be sampling at the cage level based on the location of the air filtering method, but the exhaust dust (EAD) that could be collected throughout the IVC rack system. material screening (90). Samples for rack-level air filtration can be collected by wiping the exhaust plenum directly or by screening a preliminary filter sample of the exhaust airflow. The second approach enables it to apply other commercially available systems that are slightly efficient for PCR-based testing methods (91) (92). Nonetheless, The use of gauze material (93) (94) (95) manually applied to pre-filters or cage lids or direct testing of filter top material (96) has been reported in other studies to be a successful alternative strategy.

Numerous studies have shown that EAD testing, in particular, is more

accurate than conventional sentinel programs for evaluating environmental sample material (92) (97) (98). Increasing numbers of institutions also claim that EAD testing has totally replaced the usage of sentinel animals.Particularly in accordance with the 3Rs is this paradigm change. Despite the fact that sentinel mice are typically scared during traditional HM programs (depending on the type of sample), environmental sampling can minimize or even eliminate the need for animals completely(96).

Moreover, there is adequate data that PCR-based analyzing of environmental materials actually improves the detecting effectiveness of testing procedures, which can cut down on the quantity of animals needed for research. Standardization of hygiene is substantially enhanced by this. Last but not least, environmental monitoring methods can promote animal testing by enhancing colony health generally and the avoidance of infectious diseases. EAD-based monitoring methods have been used to the following pathogens: MNV (93) (99), MHV(90), Murin Astrovirus (100), Lactate-Dehydrogenase-Elevating-Virus (LDEV) (101), *Rhodobadentibacter sp*. (83) (94), *Helicobacter sp.* (90) (99) (91), *Mycoplasma sp*. (92), *Pneumocystis sp* (92).

To date, this method has not been able to confirm infections with Klebsiella oxytoca and murine Parvoviruses (90) (91), and there are conflicting results for Staphylococcus aureus (91) (92). Subsequent research will be required to fill in this knowledge asymmetry and verify the adequacy of EAD-based monitoring for entire pathogens relating to experimental rodents, particularly for those with smaller shedding amounts, like parvoviruses.

The primary benefits of PCR-based assays include increased sensitivity of

teting and much fewer false-negative test results. As sample contamination is a common occurrence, this could be one of the disadvantages (89). A single positive PCR result should always followed by further verification of infection since false-positive results might have a severe impact on outcomes as a result of sanitary decisions (for example, population reduction of an entire colony). This is crucial since the presence of nucleic acid molecules in a pathogen does not necessarily indicate that the colony has been infected; as a result, this approach cannot differentiate between live and dead organisms and a positive result can still appear even after the presence of remaiing DNA/RNA. (17)

Washing processes of cages and overall IVC rack systems will absolutely play a crucial role in minimizing concurrent misidiagnosis because remaining nucleic acids may still be present even after routine sterilazation processes like autoclaving or irradiation (17).

In order to ensure that the colony is actually infected, positive results should always be verified conducting conventional sreening techniques, such as serological assays. In addition to the possibility of false positive results, false negative results are also possible, particularly if the gene amplification primers utilized are insufficient to identify all pertinent (sub)strains of pathogen. PCR-based assays are therefore only as efficient as its molecular design, which mostly rely on the reliability and validity of the associated datasets (17).

Especially, rapidly evolving microbes often obtain point mutations within their genomes, that could cause the failure of primer binding and subsequently in false negative results (89). As a result, it is necessary to take into consideration the pathogens' genetic drift and to constantly modify the assays that are applied to

the most recent studies. Additionally, PCR can only identify specific genetic sequences, whereas culture or microscopic methods can show all of the (cultivable) pathogens existing in the sample material and can identify dynamic changes within a population. This is very important since it could indicate a barrier leak that calls for the prompt implementation of preventive measures. Budget may also be a consideration because molecular detection methods can be costly, particularly when analyzing samples in commercial laboratories. According to some reports, however, PCR-based analysis of EAD substances is actually less costly than traditional sentinel methods (102), primarily due to the lower cost of housing. In general, using environmental sample PCR test materials is advantageous for recent HM concepts. In regards to animal welfare in specific, it should be utilized as a useful tool to supplement diagnostic methods (17).

13. Pathogen prevalence in Korean mouse facility

Microbial monitoring was performed on 21,291 mice in 206 domestic animal facilities from 2014 to 2019 to examine rodent pathogen contamination in domestic mouse facilities (Table 4). A total of 21 mouse test items were chosen based on prior infection findings (10).

Five viruses (LCMV virus, Ectromelia virus, Hantavirus, Sendai virus, Mouse hepatitis virus (MHV)) and two bacteria (C. Serologically tested (Table 5). Although MHV infection was detected in 6.1% of domestic mouse facilities, no additional viruses were found (Fig. 4). The prevalence of MHV contamination differed according to the type of institution. Companies (1.6%), hospitals (1.9%), and research institutions (3.6%) showed modest contamination rates, but universities (9.4%) had significant MHV infection rates (Figure 5) (103) .

The most common bacterial pathogen in mouse facilities was *Staphylococcus aureus* (21.2%). (Fig. 4). The prevalence of Staphylococcus aureus did not differ by institution type (Fig. 5). *Pasteurella pneumotropica* was the second most prevalent bacterial pathogen (12.5%). *Pasteurella pneumotropica* prevalence was low in hospitals (0%) and companies (1.9%) but high in universities (17.2%) and research institutions (12.5%). Pseudomonas aeruginosa was also found, but the total contamination rate was lower (5.8%); contamination rates differed by institution type, with companies having a 2.5% contamination rate, research institutes having a 9.3% contamination rate, and universities having a 6.9% contamination rate, with no contamination by these bacteria detected in hospitals (Fig. 5). There was no detection of *Salmonella spp*. or *Corynebacterium Kutscheri* (103) .

From 2014 to 2019, parasites were the most prevalent pathogens in Korean mouse facilities. The parasite infection rate varied according to the type of organization. Hospital (19.4%) and company (18.5%) facilities had comparatively small infection percentages with these test items, whereas infection rates at research institution (44.2%) and university (57.4%) facilities were significant (Fig. 5). Nonpathogenic parasite contamination was distinguished by high rates of *Octomitus intestinalis* (21.1%), *Chilomastix bettencourti* (22.1%), *Entamoeba spp.* (19.4%), and *Tritrichomonas muris* (28.7%); however, contamination with pathogenic parasites (Category C), which are not fatal but cause disease and affect physiological functions, was incredibly low (Fig. 5). The pathogenic parasite *S. muris* (2.1%) and ectoparasite *Myobia musculi* (1.3%) had lower overall infection rates than other pathogens, with the latter observed exclusively in universities. *G. muris and A. tetraptera*, the pathogenic parasites, were not identified in any of the institutions (103).

Table. 4 Type of institution and number of animals tested (103).

Type of Institution	No. of institutions	No. of animals
Hospital	16	570
Company	41	2,628
Research institute	35	3,173
University	114	14,921
Total	206	21,292

Table. 5 Test method, test items and categories

Method	Test items	Category[c]
Serological test	Lymphocytic choriomeningitis (LCM) virus	A
	Ectromelia virus	*B*
	Hantavirus	*A*
	Sendai virus	*B*
	Mouse hepatitis virus (MHV)	*B*
	Clostridium piliforme (Tyzzer disease)	C
	Mycoplasma pulmonis	*B*
Culture test	*Corynebacterium*(C) *kutscheri*	*C*
	Pasteurella(P) *pneumotropica*	*D*
	Pseudomonas(Ps) *aeruginosa*	*D*
	Salmonella spp.[a]	*A*
	Staphylococcus(S) aureus	*D*
Microscopy	*Spironucleus*(S) *muris*	*C*
	Chilomastix(C) *bettencourti*	*E*
	Entamoeba spp.	*E*
	Tritrichomonas(T) *muris*	*E*
	Octomitus(O) *intestinalis*	*E*
	Gialdia(G) *muris*	C
	Pinworm[b]	E
	Ectoparasite	C

a) *Salmonella spp*. in includes *Salmonella typhimurium and Salmonella enterica*.

b) Pinworm includes *Syphacia obvelata* and *Aspiculuris tetraptera*.

c) Microbiological categories according to the ICLAS Monitoring Center, Central Institute for Experimental Animals. Category: A, zoonotic and human pathogens carried by animals; B, pathogens fatal to animals; C, pathogens not fatal but capable of causing disease in animals and affecting their physiological functions; D, opportunistic pathogens for animals; and E, indicator of the microbiologic status of an animal.

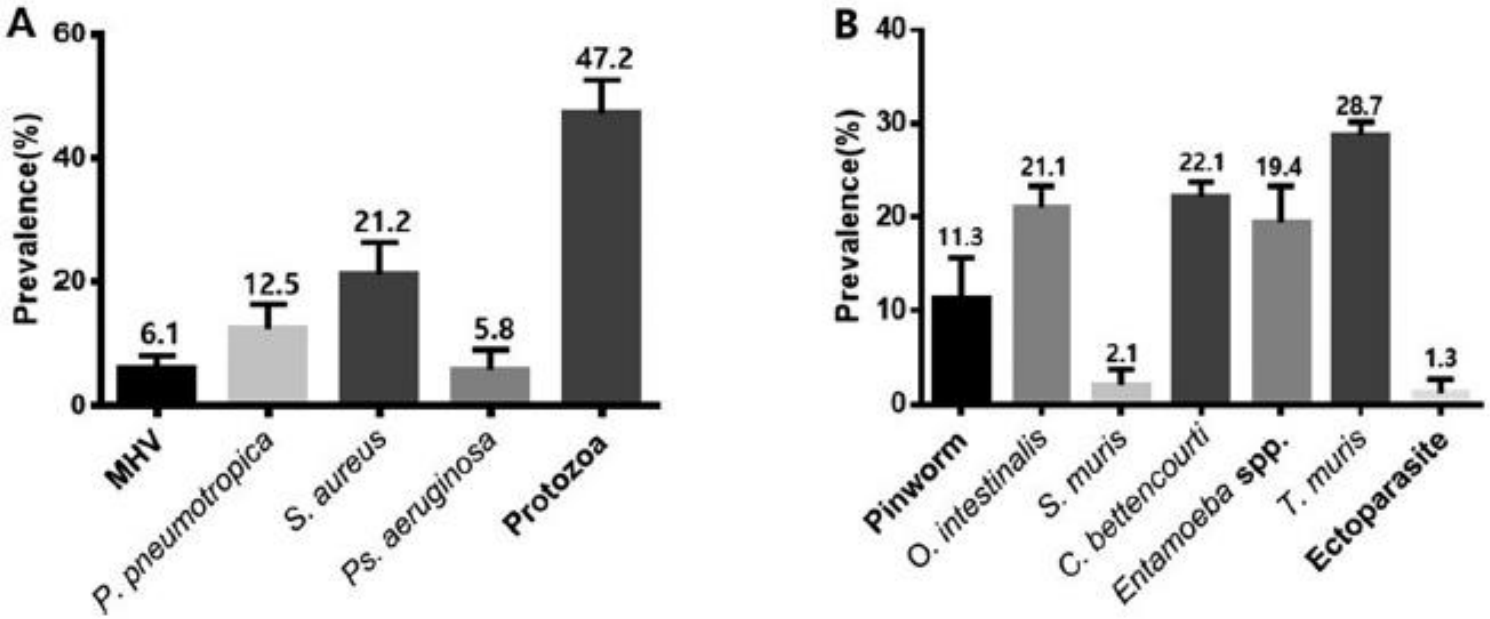

Figure 4 Microbiological contamination of mouse facilities in Korea (103).

Values shown are prevalence rates for mouse facilities. Several pathogens that were not detected are not shown in the figure A: Major pathogens contamination rates in Korean mouse facilities. B: Parasites contamination rates in Korean mouse facilities.

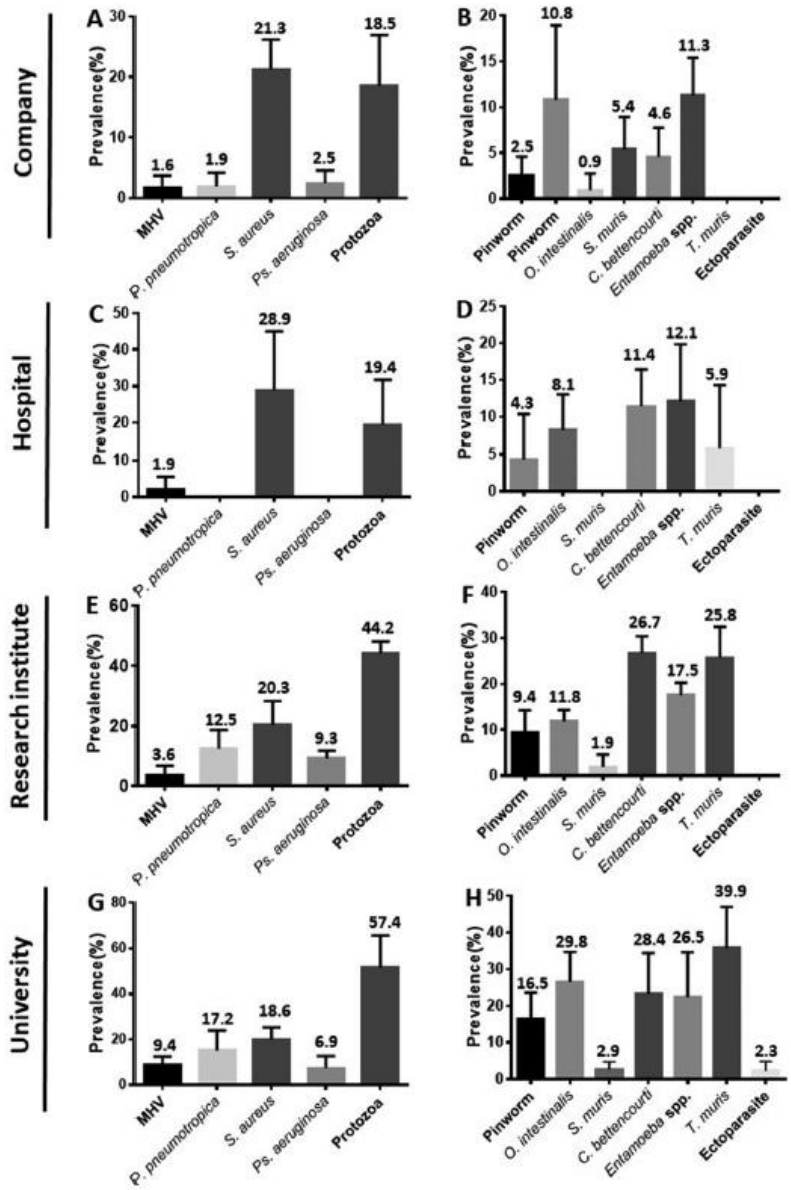

Figure 5. Microbiological contamination of mouse facilities according to the type of institution (103).

Values shown are prevalence rates for in mouse facilities. Several pathogens that were not detected are not shown in the figure. A and B: Contamination rates for major pathogens and parasites in company mouse facilities in Korea. C and D: Contamination rates for major pathogens and parasites in hospital mouse facilities in Korea. E and F: Contamination rates for major pathogens and parasites in research institute mouse facilities in Korea. G and H: Contamination rates for major pathogens and parasites in university mouse facilities in Korea. Data are presented as means ± SD (error bars).

CHAPTER I

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

1. 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 (3) (14) (104) (105) (106) (107). 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 *Pkrdcscid* mice have been reported to be highly affected by *Helicobacter spp*. (3) (56) (59) (108) (109) (110) (111) (112) (113) (114). [2, 7–15], *Trichomonas muris* infections affect the homeostasis of mucosal immune cells and susceptibility to colitis in mice (115) (116). In our laboratory mouse facility, more than 90% of the mice are genetically modified and immune-compromised. 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 the pain and distress of the animals (117). 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 (106) (118) (119). While in previous studies using embryo transfers, there was no report on whether the removal of intestinal protozoa (*Entamoeba* spp.) was successful (120) (121) (122). 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.

2. MATREIALS AND METHODS

2.1 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℃ 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.

2.2 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.

2.3 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.

2.4 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 (80mg/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℃ 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.

2.5 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.

2.6 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') (123). 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') (124). 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.

3. RESULTS

3.1 Prevalence of pathogen in sentinel mice

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.

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)

3.2 Post-rederivation Health surveillance result

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 lists of mouse strains are provided in Table 2.

Table 2 Post-rederivation Health surveillance result in mouse strains

Room	No.	Strain	Immune status[a)]	MNV		Helicobacter		Trichomonas spp.		Entamoeba spp.		No. of trans-ferred em-bryos	Total No. of pups	Method
				Sur-rogate mother	Pup	Sur-rogate mother	Pup	Sur-rogate mother	Pup	Sur-rogate 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.

3.3 Efficacy of rederivation method for MNV, Helicobacter, and Intestinal protozoa

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 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-Hdac1tm1.1Mrl/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.

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 infected pups (B6.129SV.HDAC1$^{-/-}$) were identified.

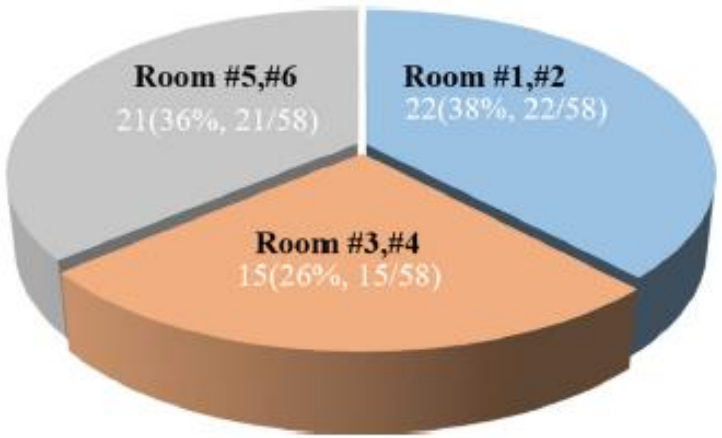

Figure 1 Prevalence of complex infection in mouse strains

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).

4. 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) (104) (106) (114). 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%) (125).

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%) (104). 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 (104) (106). 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) (10) (104). 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 (14). 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%) (106) (118) (119). 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 (119). Although MNV infection does not cause clinical symptoms in mice, combined MNV and *Helicobacter* spp. infections have been reported to accelerate the progression of

Helicobacter-related IBD in Mdr1a-null mice (56). 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 (122) (126) (127) . 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 (128). 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(106) (118) (122).

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 (106) (118). Mice have recently been microbiologically tested by RT-qPCR using dust collected by filters in individual ventilation cages. In the case of MNV, this RT qPCR method has been reported to be more sensitive than serum testing (93). 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. I used four superovulated egg donors in the embryo transfer process. 10–20 females are required for IVF and ICSI procedures (122). 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.

Chapter II

Evaluation of exhaust air dust PCR as a supplement method for soiled bedding sentinel monitoring in specific pathogen free mouse facility using two different individually ventilated cage racks

1. INTRODUCTION

Laboratory mouse health monitoring ensures animal health and quality of biomedical research results (17). The use of individually ventilated cage (IVC) rack systems in specific pathogen free (SPF) mouse facilities has lately increased. SPF mice are housed in IVC racks in the majority of mouse facilities globally, and health monitoring is implemented using a soiled bedding sentinel (SBS). A sentinel cage is usually placed every 50–80 IVCs. Because of several factors such as housing circumstances, immunological status of resident animals, and a limited number of animals for direct sampling, health monitoring using soiled bedding sentinel (SBS) is the most standard monitoring method for indirect testing of IVC-housed mouse colonies (1). Even though SBS monitoring is a standard method, it has the limitation that some pathogens are not sufficiently transmitted to sentinels housed in IVCs. Respiratory pathogens including *Faecalibaculum rodentium*, *Rodentibacter pneumotropicus, Rodentibacter heylii,* and Sendai virus have been reported to be challenging to detect by SBS monitoring (90) (94).

In previous studies, exhaust air dust (EAD) PCR monitoring was found to be a suitable detection method for several murine pathogens including *Helicobacter spp*. (90) (91) (99), lactate–dehydrogenase–elevating–virus (LDEV) (101), *Mycoplasma spp.* (92), murine astrovirus10, MHV5, MNV (93) (99), *Rodentibacter spp*. (83) (95), *Pneumocystis spp*. (92), fur mite (90) (100) (129), pinworm (90) (96) (130) and intestinal (90) (91), protozoa (91) (92) (130). However, it is unknown whether the EAD PCR test can detect all infections confirmed by SBS surveillance. For *Staphylococcus aureus*, different results have been reported (91) (92). EAD PCR failed to detect infection with murine parvoviruses or *Klebsiella oxytoca* (90) (91).

In Korea, health monitoring programs using EAD PCR have not yet been applied to laboratory animal facilities. Using SBS and EAD PCR monitoring in an SPF mouse facility, I compared the microbiological status of mouse colonies housed in the two IVC racks.

2. MATREIALS AND METHODS

2.1 Animals

BALB/c mice (four weeks old, female) were used as sentinels under SPF conditions, introduced from OrientBio (Seoul, Korea), a registered laboratory animal commercial vendor. In accordance with animal welfare principles, this animal protocol followed humane end point criteria

2.2 Husbandry

In each mouse room, a relative humidity of 40% -60%, a temperature of 22±1 ℃, and a 12:12-hour light-dark cycle were maintained. In each sentinel cage, two BALB/c mice were housed in IVCs. The irradiated mouse diet (Teklad global 18 percent protein 2018S, USA) and autoclaved reverse osmosis (RO) water were freely available to the mice. Sterilized aspen bedding (LAS bedding PG3, Germany) and enrichment materials (Ancare, USA) were provided to each cage. Used cages were replaced on a weekly basis with new ones. After briefly dipping autoclaved forceps in a disinfectant (Virkon-S, Lanxess, Germany)., mice were transferred from cage to cage.

2.3 Facility

The IBS Laboratory Animal Resource Facility has 17 mouse rooms with IVC systems. All mouse strains including genetically engineered mice were housed in a

SPF barrier. Mice from licensed vendors were quarantined before they were introduced into the main mouse colony. In vitro fertilization (IVF) has been used to rederive mice from various research institutes.

2.4 Individually ventilated cage (IVC) rack

A micro-ventilation cage system (MVCS) rack (Three-Shine. Inc., Korea) can accommodate up to 80 cages and is connected to a standing air control unit (ACU) capable of providing 60 ventilations per hour. Three-shine IVC is solid bottom polycarbonate. (20.0 × 32.0 × 14.5 cm) Air enters the cage through a supply valve linked to the cage and exits through the top filter of the cage and the exhaust valve on the lid. Before being delivered to the cage, the indoor air was filtered with a pre-filter and a HEPA filter, and the air exhausted from the cage was likewise filtered with a pre-filter and a HEPA filter before entering the mouse room exhaust system. (Figure 1)

A mouse cage system standard IVC system (Lab & Bio, Korea) can hold 80 cages, which are linked to a stand type ACU that provides 70 ventilations per hour. Lab & Bio IVC is solid-bottom polysulfonate. (19.0 × 37.2 × 13.0 cm) Air was provided and exhausted at lid height. Air dust exhausted from the cage can descend to the horizontal air-exhaust plenum via the vertical plenums. Before entering the cages, room air was filtered through the HEPA and pre-filter. After filtration with the HEPA and pre-filter, the exhaust air entered the room exhaust air system. Negative pressure was maintained in both the exhaust plenum (Three-Shine) and exhaust filter box (Lab & Bio) to prevent unfiltered air from being released. (Figure 1) The average occupancy percentage in each IVC rack was 80%.

Figure 1. Individually ventilated cage (IVC) rack

The MVCS IVC rack (Three-shine, Korea) includes a standing air control unit (AHU) and supports 80 cages with 60 air changes per hour. (A) The Lab & Bio IVC system (Lab & Bio, Korea) includes a standing ACU and can accommodate 80 cages with 70 air changes per hour. (B)

2.5 Selection of pathogen

Based on the FELASA recommendations (1) and the prevalence of pathogens in laboratory mouse facilities in Korea (103), 16 mouse pathogens, including *Staphylococcus aureus*, were chosen.

2.6 Sample collection, EAD PCR

EAD samples were taken from the IVC rack's vertical air-exhaust plenum (Three-Shine, Figure 2) and beneath the ACU's pre-filter (Lab & Bio, Figure 2) using sterile collecting media. For optimal dust accumulation, the EAD collection medium was exposed for three months. All of the samples were delivered to Charles River Laboratories (Wilmington, USA) for analysis by RT-PCR.

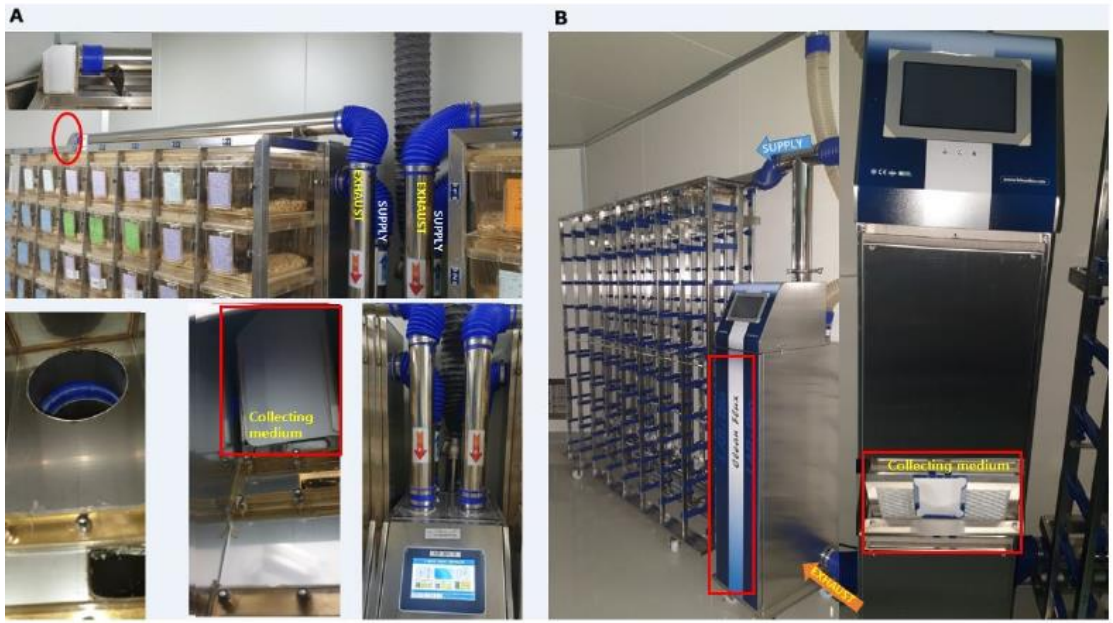

Figure 2. EAD sample collection

EAD samples were taken MVCS IVC rack's (Three-Shine, Korea) vertical air exhaust plenum (A) and beneath the ACU's pre-filter of a Mouse cage system standard IVC (Lab & Bio, Korea) (B) using sterile collecting media.

2.7 Soiled bedding sentinel (SBS) test

One BALB/c mouse from the sentinel cage placed in each single-sided IVC rack was used for SBS testing. (Figure 3) Sentinel mice were used for health monitoring after 3 months of exposure to dirty bedding collected during cage changes. Every week, each sentinel mouse cage set (cage, wire, bottle, bottle nozzle) was changed. Sentinel mice were exposed to soiled bedding and fecal materials of the resident mice colony in the same IVC rack during cage changes. Two teaspoons of soiled materials were collected from each cage on the two rows (16 cages) of the same rack on a weekly rotating system. The sentinel cage was filled with the collected soiled materials mixed with the same amount of fresh bedding. Sentinel mice were sent to Korea Research Institute of Bioscience and Biotechnology (KRIBB, Korea) for health monitoring. Serological testing for MHV, MMV, MPV, MNV, MVM, PVM, GDVII, EDIM was implemented using an ELISA kit (Charles River Laboratories, USA). *Rodentibacter pneumotropicus/heylii*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* were identified via bacterial cultures. Intestinal protozoa and, pinworms, were identified by microscopic inspection of fresh fecal samples or direct smears of the intestinal contents in cecum. *Helicobacter hepaticus, Helicobacter bilis, Helicobacter spp*. were tested by traditional PCR using cecal contents.

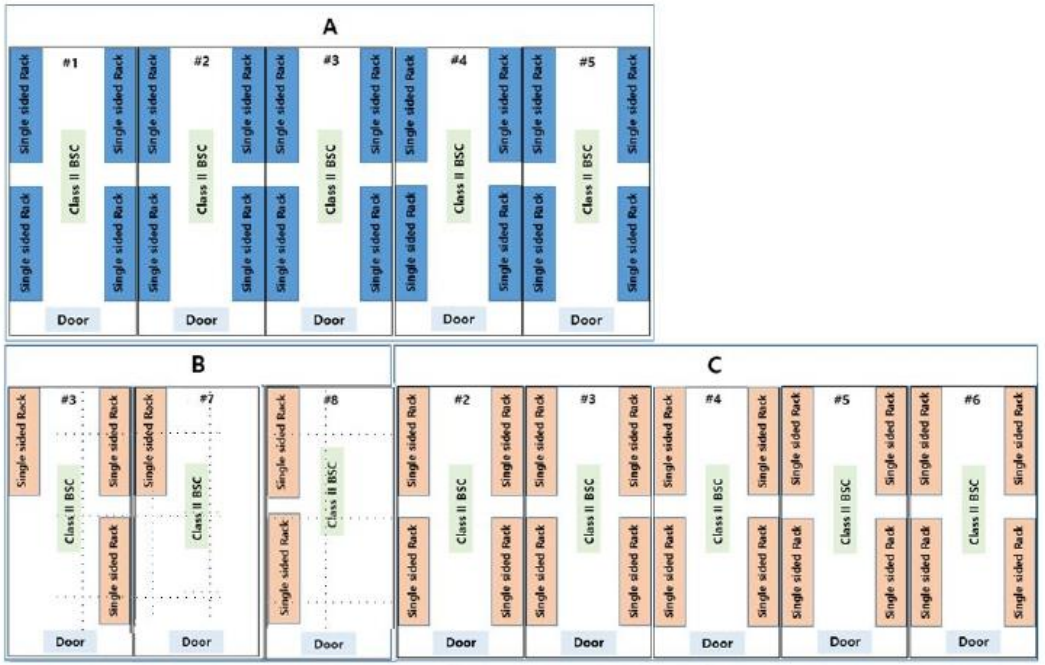

Figure 3. Arrangement of IVC racks in mouse rooms

Three-Shine IVC (A), Lab & Bio IVC (B, C), Sentinel cage was placed in each single-sided IVC rack

2.8 Pooled fecal PCR test

A maximum of ten fresh fecal pellets were pooled from each cage in the SPF mouse room. All samples were delivered to Charles River Laboratories for analysis of 16 pathogens.

3. RESULTS

3.1 Prevalence of pathogen in SPF mouse colony by Health Monitoring method

In our institution, two types of racks were used for housing mice: Three-Shine IVC racks (Area A) and Lab & Bio IVC racks (Areas B and, C), which allowed us to evaluate microbiological status using EAD PCR testing.

Sentinel mice from 13 mouse rooms in three SPF areas and EAD samples from the IVC rack were tested for 16 pathogens. *Staphylococcus aureus* was positive in two of the 44 sentinels as detected by the health monitoring test. Two out of 35 EAD samples were confirmed to be *Staphylococcus aureus* positive in the PCR test. In the 44 fecal samples, no *Staphylococcus aureus* was detected by the PCR test. Twelve out of 35 EAD samples were confirmed to be *Helicobacter spp*. positive using PCR. (Table 1)

Table 1. Prevalence of pathogen in SPF mouse colony by Health Monitoring method

Pathogen	SBS	EAD PCR	Fecal PCR
Mouse hepatitis virus (MHV)	0/44[a)]	0/35	0/44
Minute virus of mice (MVM)	0/44	0/35	0/44
Mouse parvo virus (MPV)	0/44	0/35	0/44
Mouse encephalomyelitis virus (GDVII)	0/44	0/35	0/44
Pneumonia virus of mice (PVM)	0/44	0/35	0/44
Rotavirus (EDIM)	0/44	0/35	0/44
Murine norovirus (MNV)	0/44	0/35	0/44
Rodentibacter pneumotropicus	0/44	0/35	0/44
Streptococcus pneumonia	0/44	0/35	0/44
Helicobacter hepaticus	0/44	0/35	0/44
Helicobacter bilis	0/44	0/35	0/44
Helicobacter spp.	0/44	12/35	10/44

a) Prevalence of pathogen (positive number/tested number)

3.2 Detection of Helicobacter spp. and Staphylococcus aureus by IVC Rack

A health monitoring test was implemented in ten mouse rooms in three SPF areas. In Area A, no pathogens were detected in 19 sentinels and 19 fecal samples from the five mouse rooms. Four 4 out of 20 EAD samples from Area A were detected *Helicobacter spp.* positive (20%). In Area B, *Staphylococcus aureus* was detected in one 1 out of seven 7 sentinels in three mouse rooms (14.3%). No pathogens were detected in the five EAD samples and seven fecal samples from the three mouse rooms. In Area C, *Staphylococcus aureus* was detected in one out of 18 sentinels in five mouse rooms (5.5%). Two out of ten EAD samples from Area C were positive for *Staphylococcus aureus* (20%) by PCR, and eight were detected positive for *Helicobacter spp.* (80%). Ten of 18 fecal samples were *Helicobacter spp.* positive (55.5%). (Table 2)

Table 2. Detection of *Helicobacter spp*. and *Staphylococcus aureus* by IVC rack

Area A											
IVC Rack (Three shine)		**A-1-1**	**A-1-2**	**A-2-1**	**A-2-2**	**A-3-1**	**A-3-2**	**A-4-1**	**A-4-2**	**A-5-1**	**A-5-2**
Helicobacter spp.	EAD (4/20)	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	2/2	2/2
	SBS (0/19)	0/2	0/2	0/2	0/2	0/2	0/2	0/1	0/2	0/2	0/2
	Fecal (0/19)	0/2	0/2	0/2	0/2	0/2	0/2	0/1	0/2	0/2	0/2
Staphylococcus aureus	EAD (0/20)	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	SBS (0/19)	0/2	0/2	0/2	0/2	0/2	0/2	0/1	0/2	0/2	0/2
	Fecal (0/19)	0/2	0/2	0/2	0/2	0/2	0/2	0/1	0/2	0/2	0/2

Helicobacter spp.: EAD(4/20, 20%), Fecal(0/19,0%), SBS(0/19, 0%)
Staphylococcus aureus : Not detected

Area B						
IVC Rack (Lab & Bio)		**B-3-1**	**B-3-2**	**B-7-1**	**B-8-1**	**B-8-2**
Helicobacter spp.	EAD (0/5)	0/1	0/1	0/1	0/1	0/1
	SBS (0/7)	0/2	0/2	0/1	0/1	0/1
	Fecal (0/7)	0/2	0/2	0/1	0/1	0/1
Staphylococcus aureus	EAD (0/5)	0/1	0/1	0/1	0/1	0/1
	SBS (1/7)	0/2	0/2	0/1	1/1	0/1
	Fecal (0/7)	0/2	0/2	0/1	0/1	0/1

Helicobacter spp.: Not detected
Staphylococcus aureus: SBS (1/7, 14.3%), Not detected in EAD and fecal PCR

Area C											
IVC Rack (Lab & Bio)		**C-2-1**	**C-2-2**	**C-3-1**	**C-3-2**	**C-4-1**	**C-4-2**	**C-5-1**	**C-5-2**	**C-6-1**	**C-6-2**
Helicobacter spp.	EAD (8/10)	1/1	1/1	0/1	0/1	1*/1	1/1	1*/1	1/1	1/1	1/1
	SBS (0/18)	0/2	0/2	0/1	0/1	0/2	0/2	0/2	0/2	0/2	0/2
	Fecal (10/18)	2/2	2/2	1/1	1/1	2/2	2/2	0/2	0/2	0/2	0/2
Staphylococcus aureus	EAD (2/10)	1/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
	SBS (0/18)	0/2	0/2	0/1	0/1	1/2	0/2	0/2	0/2	0/2	0/2
	Fecal (0/18)	0/2	0/2	0/1	0/1	0/2	0/2	0/2	0/2	0/2	0/2

Helicobacter spp.: EAD (8/10, 80%), Fecal (10/18, 55.5%), SBS (0/18, 0%) "+*" shows *Helicobacter ganmani*
Staphylococcus aureus: EAD (2/10, 20%), Fecal (0/18, 0%), SBS (1/18, 5.5%)

3.3 Helicobacter species specific assays

Helicobacter species specific assays were performed on EAD and fecal samples detected positive for *Helicobacter spp.* in Areas A and C. Two EAD samples in Area C were identified as *Helicobacter ganmani.* (Table 3)

Table 3. *Helicobacter species* specific assays

Sample	Estimated copy No./ Reaction	
	Helicobacter genus	*Helicobacter ganmani*
A-5-1	50	Unidentified
A-5-2	50	Unidentified
C-2-1	3275	Unidentified
C-2-2	6579	Unidentified
C-4-1	811	201*
C-4-2	404	Unidentified
C-5-1	811	201*
C-5-2	1630	Unidentified
C-6-1	1630	Unidentified
C-6-2	100	Unidentified

*Two EAD samples in area C were identified as *Helicobacter ganmani.*

4. DISCUSSION

SBS and EAD PCR monitoring for 16 pathogens in three SPF areas were performed in this study. An appropriate site for collecting EAD samples was chosen based on the air flow of each IVC rack, and EAD samples were collected for three months. In the Lab & Bio IVC rack, a medium for the EAD sample was placed under ACU pre-filter, while a holder was added to the vertical exhaust port of the Three-Shine IVC rack to collect EAD samples. To prevent the emission of unfiltered air, the EAD sampling sites were kept at a negative pressure. Some IVC racks were designed to optimize EAD surveillance using a specially developed medium consisting of filters and filter holders (91). The optimized EAD sample medium is available with Techniplast and Allentown's IVC. It is expected that more accurate testing will be possible if a customized EAD medium is developed for the two types of IVC systems used.

In this study, mouse parvovirus, mouse norovirus, mouse hepatitis virus, mouse rota virus, Theiler's murine encephalomyelitis virus (TMEV), *Rodentibacter pneumotropicus, Rodentibacter Heylii,* Flumites, pinworms, *Giardia*, *Spironucleus*, and *Tritrichomonas* were not detected by SBS and EAD PCR monitoring. In areas A and C *Helicobacter spp.* were not detected by the SBS test, whereas they were detected by EAD PCR in 40% of the test samples. This study confirmed that the EAD PCR test was more sensitive to *Helicobacter spp*. than SBS, as previously reported in several studies. The inability of the SBS test to detect *Helicobacter spp*. is thought to be due to the current state of infection. The EAD PCR was used to analyze air dust samples collected from a mouse colony kept in an IVC rack for three months, and DNA debris of *Helicobacter spp*. were detected in the accumulated

samples.

Helicobacter spp. were found in ten EAD samples from two different locations. Following *Helicobacter* species specific tests on ten positive samples, it was determined that two of them were *Helicobacter ganmani.* Previous study have found *Helicobacter ganmani* in IL10-deficient mice (131). *Helicobacter ganmani* is associated with changes in inflammatory cytokines in IL10-deficient mice. In studies using immune-deficient mice including SCID, NOD, NSG and humanized mice, infection with *Helicobacter ganmani* requires caution. EAD PCR revealed the presence of *Helicobacter mastomyrinus*, *Helicobacter ganmani, Helicobacter hepaticus,* and *Helicobacter typhlonius* in a C57BL/6 background mouse colony (91). *Helicobacter spp*. were found using SBS test in a previous study, and additional identification of positive samples indicated presence of *Helicobacter mastomyrinus*. EAD samples, on the other hand, indicated presence of *Helicobacter mastomyrinus*, *Helicobacter. ganmani, Helicobacter. hepaticus, and Helicobacter typhlonius* (99). In our facility, mice were routinely moved from Room 5 of Area A to rooms of Area C on a scale of five cages three times a week. These transportations were closely related to the prevalence of *Helicobacter spp*. in Area C. *Helicobacter spp.* were spread to the mouse rooms in Area C by mice transported from Room 5 in Area A. Two *Helicobacter ganmani*-positive EAD samples were collected from the IVC racks of rooms 4 and 5 in Area C. The mouse colonies in these racks were immune-competent, and *Helicobacter* PCR results from SBS monitoring were negative.

Regular health surveillance using EAD PCR is required for mouse studies such as hepatitis and inflammatory bowel disease (IBD) affected by *Helicobacter spp.* The detection of *Helicobacter spp*. in the Tecniplast and Allentown IVC racks

did not differ from the previous study (91). Similar to the previous study *Helicobacter spp*. were found in EAD samples from the two types of IVC racks used in this study. 2 out of 44 sentinels tested positive for *Staphylococcus aureus* (B-8-1, C-4-1). In EAD samples from IVC racks where sentinels were positive for *Staphylococcus aureus*, no *Staphylococcus aureus* was identified. In EAD samples from other areas, no 2 *Staphylococcus aureus* positive samples were found (C-2-1, C-2-2). Four positive results for *Staphylococcus aureus* in either SBS or EAD PCR test were confirmed in Areas B and C. However, the SBS and EAD PCR results for *Staphylococcus aureus* did not match in the same IVC rack. In Area B (B-8-1, C-4-1), the low copy number of *Staphylococcus aureus* in EAD samples from naturally infected immuno-competent mouse colonies can be difficult to identify using PCR. In previous studies, *Staphylococcus aureus* was not detected in EAD samples from two different IVC racks where mice infected with *Staphylococcus aureus* were housed. The low number of copies of *Staphylococcus aureus* has been identified as the reason for this finding (91). In one study, both SBS and EAD PCR monitoring detected *Staphylococcus aureus* (92). In this study PCR tests were performed on pooled fresh fecal samples (one pellet from each of the 10 cages), but *Staphylococcus aureus* was not detected.

Opportunistic bacteria (OB), including *Staphylococcus aureus*, can be challenging to detect because they are usually found in low copy numbers in naturally infected immune-competent animals. Although PCR is a more sensitive approach than culture, it does not always identify OB in animals from a known-positive colony. It is more difficult to detect intermittent or poorly excreted pathogens in infected mice using PCR testing of pooled fecal samples (89). Our

result showed that two tests should be performed in conjunction with one another to accurately detect *Staphylococcus aureus*. Further study is required to detect *Staphylococcus aureus* reliably using EAD PCR. This study focused on Three-Shine and Lab &Bio IVC racks. For several mouse pathogens, EAD PCR monitoring of these two different IVC racks requires additional study.

Some authors have reported that EAD PCR analysis has limitations due to cross-reactivity with the PCR assay used and false positives (132). Furthermore, the capability to detect contaminated trace DNA fragments or inactive substances in the environment has been identified as a result of the high sensitivity of EAD PCR surveillance (94) (97). A PCR test can result in false-positive owing to sample contamination or non-specific amplification of DNA (1). In addition, nucleic acids of pathogens may remain in all environments within the laboratory animal facility. Some authors have reported that MNV and *Helicobacter spp*. were detected in samples collected from the pre-filters of the bedding disposal cabinet where MNV and *Helicobacter spp*. were endemic (133). Prior to further testing, it is critical to properly clean and decontaminate the rack, since any leftover nucleic acid residues within the IVC rack component can be collected and cause false-positive results.

In previous studies, the EAD PCR test was found to be comparable to the SBS test or more effective for certain pathogens (92) (99). When compared to the SBS test, the EAD PCR test showed similar results in this study. In particular, the EAD PCR test had a higher sensitivity to *Helicobacter spp*. In a recent study, a filter material was placed in an IVC rack, and the dirty bedding was exposed every other week for three months without sentinel mice, followed by a PCR test after shaking for 15 seconds twice a week to generate an aerosol from the bedding. This study

showed that PCR results from the filter material were effective for detecting MNV, *Helicobacter spp., Rodentibacter spp., Spironucleus muris*, E. coli., *Spironucleus muris, Rodentibacter spp*., and *Entamoeba muris.* (134).

Some authors have compared the financial aspects of health monitoring between SBS and EAD PCR. According to them, EAD PCR was 26% less costly than SBS monitoring. To compare the cost benefit of two methods, a detailed expense analysis for the overall test scale of individual mouse facilities, calculation of additional costs for both animal test in the event of an infection outbreak and further tests for the potential false positive results of EAD PCR are required. Aside from financial benefits, the working time spent on health monitoring was lowered by EAD. Furthermore, no sentinel mice were used in the EAD PCR (102).

Thus, EAD PCR can supplement SBS monitoring, enhancing microbiological quality control in animal facilities. EAD PCR can reduce the number of animals used, making it a 3R-consistent method. If further validation for several pathogens such as *staphylococcus aureus* is performed in the future, EAD PCR can completely replace SBS monitoring.

5. GENERAL 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 wildtype 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, I 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.

I compared SBS and EAD PCR monitoring in an SPF mouse facility, with respect to detection of microbiological status of mouse colonies housed in two IVC racks. In the detection of *Helicobacter spp*., EAD PCR was more sensitive than SBS. *Helicobacter spp*. were not detected using SBS monitoring. This study focused on Three-Shine and Lab &Bio IVC racks. According to our findings, EAD PCR can be used as a supplement to SBS monitoring. Moreover, EAD PCR can reduce the

number of animals used, making it a 3R-consistent method.

In conclusion, the elimination of infectious pathogens using embryo transfer and regular microbiological monitoring using SBS and EAD PCR are crucial for the quality control of the SPF mouse facility. The reliability of animal test results and 3R compliance can be assured through quality control of SPF mouse facilities.

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국문 초록

수정란 이식과 배기 공기 분진 중합효소 연쇄반응을 이용한 특정 병원체 부재 마우스 시설의 정도 관리

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병원체는 면역이 저하된 동물과 유전자 조작 마우스에서 생리학 및 면역학적 반응에 영향을 미칠 수 있다. 특히, 마우스 노로바이러스 (Murine norovirus, MNV), *Helicobacter spp.* 및 장내 원충의 감염은 전 세계 설치류 실험실 시설에서 널리 퍼져 있다. 본 연구에서, 오염된 깔짚에 노출시킨 센티넬 마우스 (Soiled bedding sentinel, SBS)에 대한 미생물모니터링 검사 결과, 마우스 노로 바이러스 MNV (50.9%, 28/55), *Helicobacter hepaticus* (29.1%, 16/55), *Trichomonas spp.* (14.5%, 8/55) 및 *Entamoeba spp.* (32.7%, 18/55)의 감염이 확인되었다. 모든 증례에서 2개 또는 4개의 병원체가 복합 감염된 것으로 확인되었으며 단일 병원체 감염은 없었다. 선행 연구에서 교차 양육 (Cross fostering)을 이용한 청정화 방법은 성공률이 완벽하지 않았다. 따라서 이 연구에서는 SPF 설치류 시설의 전체 마우스 계통들을 수정란 이식 방법으로 청정화를 진행하였다. 청정화 완료 후 최대 3년 동안 정기적인 미생물모니터링 검사에서 검사결과가 음성 임을 확인했다. 따라서 수정란 이식은 특정 병원체 부재 (Specific pathogen free, SPF)의 배리어 마우스 시설의 청정화를 위한 효과적인 방법으로 확인되었다. 이것은 전체 SPF 마우스 시설에서 복합 감염이 있는 마우스 집단을 청정화 시킨 성공적인 사례로서 수정란 이식의 유효성에 대한 보고이며 수정란 이식은 청정화에 이용할 수 있다.

동물의 건강과 신뢰할 수 있는 연구 결과를 보장하기 위해서는 건강

모니터링이 필수적이다. 각 동물 시설은 적절한 미생물모니터링 방법을 수립하고 정기적인 미생물 검사를 통해 미생물학적 품질 관리를 실시해야 한다. 최근 특정 병원체 부재 (SPF) 마우스는 전 세계적으로 대부분의 마우스 시설에서 개별 환기 케이지 (Individually ventilated cage, IVC) 랙에서 사육되고 있으며, 미생물모니터링 검사는 오염된 깔짚 센티넬 (SBS) 마우스를 사용하여 시행되고 있다. SBS 모니터링은 표준적인 검사방법이지만 일부 병원체는 개별 환기 케이지 (IVC)에 사육중인 센티넬 마우스에 충분히 전달되지 않는다는 한계가 있다. 배기 공기 내 분진 중합효소연쇄반응 검사는 (Exhaust Air Dust, EAD PCR) 연구 결과들을 바탕으로 SBS 모니터링에 대한 신뢰할 수 있는 보완 방법으로 보고되고 있다. 국내에서는 아직 EAD PCR을 이용한 미생물모니터링 프로그램이 실험동물 시설에 적용되지 않고 있다. 본 연구에서는 두 종류의 개별 환기 케이지 (IVC) 랙에 사육된 마우스 집단의 미생물학적 상태를 특정 병원체 부재 (SPF) 마우스 시설에서 SBS 및 EAD PCR 모니터링을 사용하여 비교했다. 헬리코박터 종 (Helicobacter spp.)과 황색 포도상구균 (Staphylococcus aureus)를 제외한 16개 병원체의 검출은 두 방법 간에 차이가 없었다. 헬리코박터 종의 검출에서는 EAD PCR이 SBS보다 더 민감한 것으로 확인했다. 헬리코박터 종은 SBS 검사에서는 검출되지 않았지만, 4개의 황색포도상구균 양성 샘플이 SBS 또는 EAD PCR 검사에서 확인되었다.

우리의 연구 결과에 따르면 EAD PCR은 SBS 모니터링의 보완 검사법으로 사용될 수 있다. 또한 EAD PCR은 사용하는 동물의 수를 줄일 수 있어 3R (동물실험 대체법, 동물 사용수 최소화, 통증 관리)에 부합하는 방법이다.

결론적으로 수정란 이식을 통한 감염성 병원체의 제거와 오염된 침구 센티넬 (SBS) 및 배기 공기 내 먼지 (EAD, Exhaust Air Dust) 중합효소연쇄반응 (PCR)을 사용한 정기적인 미생물모니터링 검사는 특정 병원체 부재 마우스 시설의 품질 관리에 필수적이다. 특정 병원체 부재 마우스 시설의 품질 관리를 통해 동물 실험 결과의 신뢰성을 확보하고 3R (동물실험 대체법, 동물 사용수 최소화, 통증 관리)을 재현할 수 있을 것으로 사료된다.

주요어: 특정 병원체 부재 마우스, 병원체, 수정란 이식, 청정화, 오염 침구 센티넬 마우스 미생물모니터링, 배기 공기 분진 중합효소 연쇄반응

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