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Health Surveillance of Specific Pathogen-Free and Conventionally-Housed Mice and Rats in Korea

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Abstract: *The present study contains information about proper microbiological monitoring of laboratory animals' health and the standardization of microbiological monitoring methods in Korea. Microbiological quality control for laboratory animals, composed of biosecurity and health surveillance, is essential to guard against research complications and public health dangers that have been associated with adventitious infections. In this study, one hundred and twenty-two mice and ninety rats from laboratory animal breeding companies and one animal facility of the national universities in Korea were monitored in 2000–2003. Histopathologically, thickening of the alveolar walls and lymphocytic infiltration around the bronchioles were observed in mice and rats from microbiologically contaminated facilities. Cryptosporidial oocysts were observed in the gastric pits of only conventionally-housed mice and rats. Helicobacter spp. infection was also detected in 1 of 24 feces DNA samples in mice and 9 of 40 feces DNA samples in rats by PCR in 2003, but they were not Helicobacter hepaticus. This paper describes bacteriological, parasitological, and virological examinations of the animals.*

Key words: *cryptosporidial oocysts, helicobacter spp., laboratory animals, microbiological monitoring*

Introduction

It has been amply documented that adventitious infections of laboratory animals with certain microorganisms can interfere with research. Infections may result in clinical disease and pathological changes, especially in perinatal and immunodeficient animals [3, 12, 18, 32, 33, 35, 37]. Furthermore, some microorganisms indigenous to laboratory animal species are zoonotic agents that have caused disease in humans [1, 8, 17, 24, 25]. It is therefore essential for laboratory animal breeders and users alike to implement and maintain a microbiological quality control program that includes strict biosecurity and comprehensive microbiological or health surveillance [19].

In this study, we monitored one hundred and twenty-

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Table 1. Composition of mice and rats used in this study (2000–2003)

Group	Strains	No. of animals examined		
		4 weeks old	10 weeks old	24 weeks old
A	BALB/c mice	4	4	–
	SD rats	4	4	2
B	BALB/c mice	4	4	–
	SD rats	4	4	2
C	BALB/c mice	4	4	–
	SD rats	4	4	2
D	SD rats	4	4	2
E	C3H mice	11	11	4
	C57BL/6 mice	11	11	4
	BALB/c mice	11	11	4
	ICR mice	11	11	7
	SD rats	11	11	7
	Wistar rats	11	3	7

Group A, B and C represent SPF facilities, while D and E represent conventional facilities. SD: Sprague Dawley.

two mice and ninety rats from four laboratory animal breeding companies and one animal facility of the national universities in Korea in 2000–2003. This paper focused on increasing the quality of hygiene of laboratory animals and the standardization of microbiological monitoring in Korea. To assure the microbiological quality of these animals, the population and its environment need to be screened thoroughly on a routine basis. The present investigation was conducted for the purpose of surveying the quarantine status in animal facilities and its attendant problems, and selecting the range of investigated viruses and bacteria, and confirming the microbiological monitoring methods in use in Korea.

Materials and Methods

Animals and specimen collection

One hundred and twenty-two mice and ninety rats were purchased from four major laboratory animal breeding companies from the size and facility aspect and one animal facility of the national universities in Korea (A–E: A, B, C were SPF facilities, while D and E were conventional facilities). Table 1 shows the constitution of the mice and rats used in this study. Animals were sacrificed by exsanguination under deep ether anesthesia. Samples of blood, liver, kidney, spleen, lung and colon feces were collected immediately. The feces were frozen at –20°C for PCR assays, and liver, kidney, spleen and lung samples were smeared on 5% sheep blood agar (Komed, Seoung-Nam, Korea) for bacterial detection. Blood was centrifuged at 2,000 to 3,000 rpm for approximately fifteen minutes and the serum was transferred to separate vials for ELISA. All organs were fixed in 10% neutral formation for at least 24 h for histopathological examination. All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Seoul National University.

The external surface examination for external parasite detection

Samples were collected from each anal surface using the Scotch cellophane tape method. Each cellophane tape containing anal surface samples was pressed against the surface of a glass slide. The specimens from each sample were examined microscopically for the presence of *Syphacia* spp. eggs. If suspicious lesions were detected, the tissues were processed for histopathology examination.

Nasal cavity and upper respiratory airway examination

Mucous in the nose (nasal cavity), the back of the nasal cavity (nasopharynx), the back of the mouth and tongue (oropharynx) and the upper part of the voice box (laryngo-pharynx) were collected by the insertion

Table 2. Microbiological monitoring of the health condition of mice and rats with the range of investigated viruses and mycoplasma

Viruses	
Mouse	Rat
Minute virus of mice (MVM)	Mouse adenovirus (MAD)
Reovirus type 3 (REO)	Encephalitozoon cunicali (ECUN)
Ectromelia virus (ECTRO)	Hantavirus (HANT)
Lymphonotic choriomeningitis virus (LCM)	Kilham's rat virus (KRV)
Pneumonia virus of mice (PVM)	Toolan's H-1 virus (H-1)
Mouse hepatitis virus (MHV)	Parvovirus (rNS)
Mouse polio virus (GD-7)	Reovirus type 3 (REO)
K virus	Rat coronavirus (RCV)
Mouse cytomegalovirus (MCMV)	Sendai virus
Epizootic diarrhea of infant mice virus (EDIM)	
Polyoma virus (POLY)	
Mouse adenovirus (MAD)	
Encephalitozoon cunicali (ECUN)	
Parvovirus (mNS)	
Lymphocytic choriomeningitis virus (LCMV)	
Sendai virus	
Mycoplasma	

and rotation of a small absorbent cotton swab and smeared on PPLO agar (Difco, Detroit, USA). The plates were incubated at 37°C in 5% CO_2 for 1–2 days. Isolated bacteria were identified using an automatic identification apparatus (VITEK Co., Hazelwood, USA).

Internal Parasite Examination from the fecal samples

Approximately one gram of feces collected from the large intestine was mixed with water in a tube. Liquid was strained out of the solid waste using a tea strainer. A concentrated solution of sucrose (Sigma, Saint Louis, USA) was added, the sediment stirred and the tube inverted several times. The surface layer was transferred to a microscope slide using a glass. Samples were examined soon after the slides were made to avoid crystal formation from media evaporation obscuring any ova.

ELISA for viruses and mycoplasma

The serum ELISA was performed in 96-well, polystyrene, flat-bottom microtiter plates, using an ELISA kit (Charles River, Wilmington, USA) following the manufacturer's recommended protocol scoring system. In brief, each well of an ELISA plate was coated with each antibody. After washing with PBS (pH 7.4), the plate was blocked by treatment with 1% bovine serum albumin at 4°C for 2 h, incubated with the test sera (1 in 60 dilution) at 40°C for 40 min, and washed three times with PBS-Tween. Plates were then incubated with diluted conjugate added to all wells at 40°C for 40 min. After washing, the plates were visualized with o-phenylenediamine dihydrochloride (Sigma, Saint Louis, USA) and the absorbance was measured at 450 nm. The optical density (OD) readings from the ELISA reader were conveyed to a PC where they were converted to scores by dividing by 0.13. A net score of 0 and 1 was deemed negative. Net scores of 2 and ≥ 3 were deemed borderline and positive, respectively. The net score = $\text{Score}_{\text{Antigen}} - \text{Score}_{\text{Tissue Control}}$. Table 2 is an overview of the viruses and mycoplasma examined. In case of borderline, a visual scoring method was added through visually reading color development Charles River Scoring System as follows: 0 none, 1 slight, 2 moderate, 3 intense. Then, if the antigen score was 0–1 or 2–3, the interpretation was negative or positive, but only if tissue control were < 1 (if tissue control was >1, we considered this was a non-specific result).

Bacteria examination

Liver, kidney, spleen and lung samples were smeared on 5% sheep blood agar (Komed, Seoung-Nam, Korea) for bacteria examination. The plates were incubated at 37°C for 1–2 days. Isolated bacteria were identified

Table 3. Microbiological monitoring of the health condition of mice detailing the positive detection of investigated viruses and mycoplasma (2000–2003)

Animals		No. of positive samples/no. of samples tested (no. of positive facilities/no. of facilities tested)		
		Viruses		Mycoplasma
		MHV	Sendai virus	
Mice	4 weeks old	4/56 (1*/5)	6/56 (2*/5)	3/56 (1*/5)
	10 weeks old	4/56 (1*/5)	12/56 (2*/5)	2/56 (1*/5)
	24 weeks old	2/19 (1*/1)	19/19 (1*/1)	3/19 (1*/1)

*represents a conventional facility.

using an automatic identification apparatus (VITEK Co., Hazelwood, USA).

DNA extraction and PCR amplification for Helicobacter *spp. and* Helicobacter hepaticus *infection examination*

For the isolation of total genomic DNA, the colon feces samples were wrapped with foil, quickly frozen in liquid nitrogen, and powdered by striking with a hammer. The samples were then suspended in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, SDS 0.5% w/v, pH 8.0) with 100 μg of proteinase K. After incubation of the lysates at 56°C for 3 h, the DNA was extracted from each sample with phenol-chloroform and precipitated in isopropyl alcohol with sodium acetate. Each DNA sample was washed twice in 70% alcohol and resuspended in Tris-EDTA (TE: 10 mM Tris, 15 mM EDTA, pH 8.0) buffer. For the PCR assay, primers (F, 5'- CTATGACGG GTATCCGGC-3'; R, 5'- TGGGAGAGGTAG GTGGAAT-3') and (F, 5'- GAAACTGTTACTCTG-3'; R, 5'- TCAAGCTCCCCGAAGGG-3') were designed from the 16S rRNA gene of *Helicobacter* genus-specific conserved regions [31] and of *Helicobacter hepaticus*-specific conserved regions [4]. The positive DNAs were extracted from *Helicobacter hepaticus* (ATCC51448). The PCR mixture (100 μl) contained 2 μg of DNA, 100 pmol of each primer, 1 × PCR buffer with $MgCl_2$, 200 μM dNTP, and 2.5 units of Taq polymerase (PCR core Kit, Boehringer Mannheim, Germany). PCR conditions were as follows: 3 min of denaturation at 95°C, followed by 35 cycles consisting of 30 s of denaturation at 94°C, 30 s of annealing at 53°C, and 1 min of extension at 72°C, then 7 min of extension at 72°C after amplification. The PCR product was detected by the electrophoresis of 12 μl of reaction solution in 1.5% agarose gel containing 1 μg/ml ethidium bromide.

Histopathological examination

Fixed tissues were dehydrated in an alcohol-xylene series, and embedded in paraffin wax. From each block, 2 μm sections were prepared and stained with hematoxylin and eosin (HE) and modified Steiner's silver stain [29] for histopathological examination and for the detection of tightly coiled spiral bacteria, respectively.

Results

Internal and external parasites examination

Pinworms were only observed in one conventionally-housed rat.

ELISA for viruses and mycoplasma examination

In the mice, Mouse hepatitis virus (MHV), Sendai virus, and mycoplasma were detected. Both MHV and mycoplasma were detected in samples from one conventional facility. Sendai virus was detected in samples from the conventional facilities (Table 3). In the rats, Kilham's rat virus (KRV) was only observed in samples from only one of the SPF facilities. The other viruses and mycoplasma were only detected in the conventionally-housed rats (Table 4).

Bacteria examination

In the incubated plates of the mucous of the nose (nasal cavity), the back of the nasal cavity (nasopharynx), the back of the mouth and tougue (oropharynx), the upper part of the voice box (laryngo-pahrynx), liver, kidney, spleen, and lung samples, no pathogenic bacteria were detected.

Table 4. Microbiological monitoring of the health condition of rats detailing the positive detection of investigated viruses and mycoplasma (2000–2003)

Animals		No. of positive samples/no. of samples tested (no. of positive facilities/no. of facilities tested)					
		Viruses					Mycoplasma
		KRV	rNS	H-1	RCV	Sendai virus	
Rats	4 weeks old	1/16 (1**/4)	0/16 (0/4)	0/16 (0/4)	0/38 (0/5)	1/38 (1*/5)	3/38 (1*/5)
	10 weeks old	0/16 (0/4)	3/16 (1*/4)	1/16 (1*/4)	3/30 (1*/5)	2/30 (1*/5)	4/30 (1*/5)
	24 weeks old	0/8 (0/4)	0/8 (0/4)	0/8 (0/4)	5/22 (2*/5)	6/22 (2*/5)	2/22 (1*/5)

*and **represent a conventional facility and SPF facility, respectively.

Table 5. Detection of *Helicobacter* spp. and *Helicobacter hepaticus* from feces by PCR with genus-specific primers (2003)

Group	Strains	No. of PCR positive animals of *Helicobacter* spp (no. of PCR positive animals of *Helicobacter hepaticus*)/no. of animals examined		
		4 weeks old	10 weeks old	24 weeks old
A	BALB/c mice	1(0)/4	0/4	–
	SD rats	1(0)/4	0/4	0/2
B	BALB/c mice	0/4	0/4	–
	SD rats	0/4	0/4	0/2
C	BALB/c mice	0/4	0/4	–
	SD rats	0/4	0/4	0/2
D	SD rats	4(0)/4	3(0)/4	1(0)/2

Groups A, B and C represent SPF facilities and D represents a conventional facility.

PCR amplification for Helicobacter *spp. and* Helicobacter hepaticus *infection examination*

DNA was isolated from the colon feces of each mouse and rat subjected to PCR evaluation. The amplification with the *Helicobacter* genus-specific primer set showed distinct bands of the expected 374-bp size in 1 of 24 feces DNA samples from the mice housed in the SPF facility. The 374-bp amplification was detected in 8 of 10 feces DNA samples from the conventionally-housed rats (80%) and 1 of 30 feces DNA samples from the SPF facilities (3%) (Table 5). Using silver staining, bacteria were observed in the crypt of the colon. Tightly coiled bacteria were found in all infected mice and rats. Usually, larger numbers of bacteria were found in the crypts rather than near or in the lumen of the large intestine. But the amplification with the *Helicobacter hepaticus*-specific primer set showed no distinct bands of the expected 405-bp size in *Helicobacter* genus-specific positive samples (N=10).

Histopathological examination

Histopathologically, the majority of lesions presented as a thickening of the alveolar walls and lymphocytic infiltration around bronchioles in 17 of 98 mice from only the conventional facilities (17%), 4 of 30 rats from the SPF facilities (13%) and 9 of 60 rats from the conventional facilities (15%). Pericarditis was observed in only the conventional facilities, 2 of 98 mice (2%) and 2 of 60 rats (3%). Focal necrosis in the liver was observed in 1 of 24 mice from the SPF facilities (4%) and 4 of 98 mice from the conventional facilities (4%) and 4 of 60 rats from only the conventional facilities (7%). *Cryptosporidial* oocysts were observed in gastric pits in only the conventionally-housed mice (4%) and rats (6%). No other lesions were found in the stomach.

Discussion

It is clear that pathogenic agents causing overt disease represent a serious hazard. Characterization of the

health status and microbiological monitoring of the animals in experiments are particularly important for both general animal welfare and precise scientific practice. The establishment of microbiological monitoring standards, which can be used at all animal facilities of national universities and institutes, would be of great value.

In SPF facilities, MHV and mycoplasma were detected in mice and KRV was detected in rats. It was considered that each SPF facility had attempted to eradicate those diseases in their animals. The methods of eradication were as follows: a) rederivation by hysterectomy, and b) use and care in specifically designed rooms.

In the conventional facilities, the animals had a number of viruses and mycoplasma, suggesting that the facilities should take all the measures (a and b) mentioned above. Animals from these facilities should not be used as experimental animals.

From the results of the microbiological monitoring, the infection rate of viruses and mycoplasma depended on the age of the animals. As shown in Tables 3 and 4, positive results were detected more frequently in 24-week old animals than in animals of other ages. In addition, although KRV was observed in only one of the 4-week old rats, the other serologic results showed very weak borderline detections in that age group. This might indicate the maternal inheritance of viruses and mycoplasma in 4-week-old animals, because the borderline and positive serological results were also detected more strongly in older age groups. For these reasons, the older aged animals must be closely monitored.

In rodents, six *Helicobacter* species have been detected in mice (*H. hepaticus*, *H. muridarum*, *H. bilis*, *H. rodentium*, *H. rappini*, *H. typhlonius*), two in both mice and rats (*H. muridarum*, *H. bilis*), and two in rats only (*H. trogontum*, *H. heilmannii*) [10, 11, 13, 16, 23, 28, 38]. An accurate diagnosis of Helicobacter infections is needed to assess the pathogenic potential of these organisms to address concerns about their impact on research results [27]. Among the *Helicobacter* sp. which colonize the gastrointestinal tracts of rodents, only *H. hepaticus* has been clearly identified as a pathogen. In our results, Helicobacter-like organisms were histopathologically observed in the crypt of the colon (Steiner's stain) and by PCR amplification based on genus-specific 16S rRNA gene sequences. But the amplification with the *H. hepaticus*-specific primer set showed no distinct bands of the expected 405-bp size in *Helicobacter* genus-specific positive samples (N=10). The significance of these bacteria in laboratory animals needs to be understood in order to evaluate their influence on experiments and their management in rodent facilities.

At the Central Institute for Experimental Animals, serological tests [20, 21] were recommended for *Mycoplasma pulmonis*, *Clostiridium piliforme* (Tyzzer's organism), *Salmonella typhimurium*, *Corynebacterium ktscheri*, *cilia-associated respiratory* (CAR) *bacillus*, but in this study, we only performed histopathological examination. Tyzzer's disease is diagnosed most directly by the demonstration of characteristic intracellular organisms in tissue sections of the liver and intestine [9]. Asymptomatic infection can be detected by ELISA [34], or by PCR [15].

Histopathological examination demonstrated the majority of lesions presented as a thickening of the alveolar walls and lymphocytic infiltration in mice and rats from all of the facilities, indicating the Sendai virus or mycoplasma infection. In Sendai virus infection, susceptible mice usually have significant bronchopneumonia and interstitial pneumonia [19]. Mycoplasma strains can differ in virulence. Survivors of severe infection may develop chronic bronchopneumonia with bronchiectasis and spread infection to other mice. *Cryptosporidiosis* was observed in rats from the one of the conventional laboratory animal breeding facilities. In an outbreak of *cryptosporidiosis* in Warrington, a town in north-west England, 47 cases were recorded between November 1992 and February 1993 in humans by Bridgman *et al*. They reported a strong association and a dose-response relationship between having drunk unboiled tap water from these sources and *cryptosporidiosis* infection [5]. Mice and rats in particular have an intimate relationship with humans and domesticated animals and have a reputation as harbingers of disease [14]. Wild mammals, particularly rodents, have been identified as reservoirs of *C. parvum* by several authors [2, 6, 7, 22, 36]. Infections in such animals appear to be asymptomatic [30], but oocysts shed in the feces may pose a threat to human health in urban and rural environments, particularly for individuals who may have frequent contact with animal feces

such as field or farm workers. Although we confirmed the existence of *cryptosporidiosis* by histological examination, we could not find the oocysts in the feces. This was likely due to the stage of the life cycle and the number of infection organisms.

The numbers of animals used for each examination was small in this paper. For instance, only 2, 3, or 4 mice were used for some examinations (Table 1). Lindsey *et al*. recommended 10 animals from the sentinel animal population, selected in such a way as to be representative of the unit, should be subjected to a full microbiological and pathological examination [26].

When pathogenic micro-organisms are detected, the infected animals should be isolated and the staff should undertake appropriate action in order to eliminate the infection from the animals (treatment or elimination of infected subjects). One method includes isolating the litter obtained after caesarean section from the infected mother and taking them to healthy female individuals serving as surrogate mothers. Even though most infections in laboratory animals are asymptomatic, these latent diseases may be dangerous because they lead to changes in the animal immune system and they may be followed by the occurrence of clinical symptoms as a result of stress, the deterioration of environmental conditions or additional infections. This in turn may be responsible for the distortion of experiment results and/or the death of the animals. Therefore, it is recommended that the health condition of all animals be monitored at regular time intervals, usually every 3 months. In addition, the microorganisms that are tested for and the microbiological status of animals in health monitoring reports are not yet consistent among the different universities, and institutes in Korea. We propose that this microbiological monitoring become a standard for health monitoring reports of mice and rats in Korea.

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