NOTE *Laboratory Animal Science*

Prevalence of Murine Norovirus Infection in Korean Laboratory Animal Facilities

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ABSTRACT. Currently, murine noroviruses (MNV) are the most prevalent viral pathogens identified in laboratory animal facilities. While several reports exist concerning the prevalence of MNV in North American research facilities, very few reports are available for other parts of the world, including Korea. This study evaluated the prevalence of MNV infection in 745 murine sera collected from 15 animal facilities in Korea by enzyme linked immunosorbent assay (ELISA). Positive cases were subcategorized by murine strain/genetics, housing environments and animal sources. In summary, 6.6% of inbred/outbred mice purchased from commercial vendors were seropositive, 9.6% of in-house colonies were seropositive and 27.0% of genetically modified mice (GMM) were seropositive. Partial gene amplification of fecal isolates from infected animals showed that they were homologous (100%) with MNV-4.
KEY WORDS: genetically modified mouse, Korea, murine norovirus, prevalence.

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Noroviruses, members of the family *Caliciviridae*, have been associated with infection and disease in humans and mice [2, 6]. In humans, the virus causes more than 90% cases of nonbacterial epidemic gastroenteritis worldwide [6]. Transmission of human norovirus occurs via a fecal-oral route. Human norovirus is classified as a class B bioterrorism agent because the virus is highly contagious, infectious at low doses, extremely stable in the environment and associated with debilitating illness [8]. Despite the impact of norovirus-induced disease, the pathogenic features of infection are not well understood because of the inability to culture the virus *in vitro* and the lack of a small animal model system to study it [1]. Infection of mice with norovirus was first recognized and described in a colony of *Rag2/Stat*$^{-/-}$ mice [6]. In a 2005 report, the seroprevalence of this virus in a large number of murine research colonies in both the U.S.A. and Canada was found to be 22.1% [5]. Other reports made in 2008 and 2009 indicated that the seroprevalence was more than 30% [3, 9]. Currently, murine norovirus (MNV) is the most prevalent viral pathogen identified in laboratory animal facilities, and the full effect of the disease on murine research models in not yet known [5]. MNV infection has been shown to be lethal in certain immunodeficient strains of mice with defects in innate immunity, including mice with *Rag2*$^{-/-}$*/Stat1*$^{-/-}$, *Stat1*$^{-/-}$, *Stat1*$^{-/-}$*/Pkr*$^{-/-}$ and *IFNαβγ*R$^{-/-}$ genotypes [6]. However, MNV is not associated with clinical illness or death in experimentally-infected immunocompetent inbred and outbred strains of mice, or in mice with defects in acquired immunity such as *Rag 1*$^{-/-}$ or *Rag 2*$^{-/-}$ [6].

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While several reports exist concerning the prevalence of MNV in North American research facilities, very few reports are available characterizing the prevalence of infection in other parts of the world, including Korea. The purpose of this study was to evaluate the prevalence of MNV infection in mice in Korea as well as the source of the infected animals.

A total of 745 mice of eleven kinds of inbred/outbred genotypes (BALB/c, C.B10-*H2*^{b}, C3H, C57BL/6, C57BL/10, DBA/1, DBA/2, FVB, NOD, NZW and ICR) and more than one hundred kinds of genetically modified strains of both sexes ranging between 4 weeks to 21 months in age were monitored microbiologically between November 2006 and September 2009. In terms of animal sources, some mice originated from commercial vendors, some were from in-house breeding colonies and some were GMM maintained in-house. The sources, housing methods and colony types for each of the 15 facilities from which animals originated are presented in Table 1.

The type of caging and bedding used varied amongst the different facilities. Animals from facilities A to E were housed in an individually-ventilated caging system (IVCS) in an environmentally-controlled room with regulated air exchange. Facilities F to J and M to O were conventionally-maintained facilities. Facilities K and L were major SPF-grade commercial vendors in Korea. In all facilities, the animal rooms were maintained at 20–24°C, with 40%–60% relative humidity, an air exchange rate of 12–18 air changes/hr and a 12:12 hour light:dark cycle. Part of facility E is a conventionally-maintained area. The mice were fed rodent chow (LabDiet, U.S.A. or Purina, Korea) and provided with autoclaved water *ad libitum*. The bedding used consisted of hardwood, corncob or paper chips. For barrier facilities, rooms had a HEPA-filtered air supply exchange with similar

Table 1. Characteristics of 13 facilities from which mouse sera were collected

Facility	Location	Caging system	Environment	Total no. of samples			
				GMM[c]	BC[d]	CM[e]	Total
A[a]	Seoul	IVCS	Barrier	51	4	74	129
B[a]	Seoul	IVCS	Barrier	205	122	4	331
C[a]	Seoul	IVCS	Barrier	15	32	43	90
D[a]	Gyeonggi	IVCS	Barrier	2	0	24	26
E[a]	Seoul	IVCS	Barrier	3	6	29	38
		Open	Conventional	5	1	0	6
F[a]	Seoul	Open	Conventional	0	0	8	8
G[a]	Seoul	Open	Conventional	0	0	3	3
H[a]	Gyeonggi	Open	Conventional	0	5	0	5
I[a]	Chungbuk	Open	Conventional	0	0	10	10
J[a]	Chungnam	Open	Conventional	0	8	0	8
K[b]	Gyeonggi	Open	Barrier	0	0	26	26
L[b]	Gyeonggi	Open	Barrier	0	0	40	40
M[b]	Gyeonggi	Open	Conventional	0	0	9	9
N[b]	Seoul	Open	Conventional	0	0	8	8
O[b]	Chungbuk	Open	Conventional	0	0	8	8
Total				281	178	286	745

a) Research Institute. b) Commercial vendor. c) GMM: genetically modified mice. d) BC: in-house breeding colony of common inbred/outbred mice. e) CM: common inbred/outbred mice purchased from vendors.

environmental parameters as previously listed. In these facilities, the mice were fed *ad libitum* with only autoclaved or irradiated feed and autoclaved drinking water, and all materials were sterilized or disinfected prior to entry into the facility. In the animal rooms, personnel wore sterile gowns over scrubs in addition to sterile gloves, masks, caps and shoe covers. Personnel passed through an air shower prior to entering the facility.

The mice were anesthetized with a tiletamine/zolazepam mixture (Zoletil 50, 30 mg/kg IP, Virbac, France) and xylazine (Rompun, 10 mg/kg IP, Bayer, Korea) and exsanguinated from the abdominal vena cava. Samples of feces and various tissues were immediately collected. During the study period, mice in facilities A to O were monitored for evidence of viral infections by ELISA, bacterial infections by culture and parasitic infections by microscopic examination. Mice from facilities A to E and K to L lacked antibodies against common mouse pathogens [mouse hepatitis virus (MHV), mouse minute virus (MMV), mouse rotavirus (EDIM), mouse parvovirus (MPV), pneumonia virus of mice (PVM), Sendai virus, mouse encephalomyelitis virus (GDVII), ectromelia virus, lymphocytic choriomeningitis virus (LCMV), mouse cytomegalovirus (MCMV), Reo-3 virus (REO 3), *Mycoplasma pulmonis, Clostridium piliforme, Streptococcus pneumoniae, Corynebacterium kutscheri, Salmonella* spp. *Yersinia pseudotuberculosis*, *Citrobacter rodentium* and *Bordetella bronchiseptica*]. *Staphylococcus aureus* was detected in 11 mice, *Pseudomonas aeruginosa* was detected in 13 mice, *Klebsiella* spp. was detected in 15 mice, *Pasteurella pneumotropica* was detected five mice and MHV was detected in four mice. Mice in facility G were seropositive for Sendai virus. In facility M, mice were infected with MHV, *Syphacia obvelata* and fur mites. In facility N, mice were seropositive for MHV and were infested with fur mites, while mice in facility O had only fur mites. The study protocol was approved by the Seoul National University Institutional Animal Care and Use Committee.

Serological screening for MNV was performed using ELISA. Antibodies against MNV were detected using a commercially available ELISA kit (Charles River Laboratories, Wilmington, MA, U.S.A.). As per the kit instructions, each well of the ELISA plate was coated with antigen (MNV GV/CR1/2005/USA recombinant baculovirus protein) or tissue control, 1:60 diluted sera were transferred onto the test plate and then control samples were added. High, low and non-immune sera were used as standard samples for each test to ensure that the assay was working properly. The high-range control was the serum that had been diluted to yield a high ELISA score, the low-range control was the serum that had been diluted to give a low ELISA score and the non-immune control was negative serum. After incubating at 35–40°C for 40 min, the plates were washed with PBS, and diluted conjugate (HRP-labeled goat anti-mouse IgG) was added. After further incubation, the plates were washed with PBS again, 100 μl of substrate (ABTS-H_2O_2) was added and the plates were incubated for an additional 40 min. Following this incubation, an SDS stop solution was added, and the absorbance was measured at 405 nm. The results were interpreted according to the Charles River Scoring System. Serum samples that had a Net Score (OD value of antigen-coated well/0.13-OD value of tissue control well/0.13) 3 were interpreted as positive. The result was interpreted as negative when the Net Score was 0 or 1 and equivocal when the Net Score was 2. Equivocal samples were repeated, and if they remained

Table 2. MNV primer sequences

Primer no.	Primer (5'–3')	Location of primer nucleotide[a)]	Amplicon size	Reference
Primer #1	F: AGCGGCCAGGATCTTGTTCC R: AAGACTCATCACCCGGGCTG	5103–5123 5631–5650	547 bp	Kim *et al.* [7]
Primer #2	F: GAATGAGGATGAGTGATGG R: AATAGGGTGGTACAAGGG	5060–5078 5435–5452	393 bp	This study
Primer #3	F: CAGATCACATGCTTCCCAC R: AGACCACAAAAGACTCATCAC	5473–5491 5638–5658	187 bp	Hsu *et al.* [4]

a) Nucleotide positions for MNV were obtained from GenBank accession number AY228235.

Table 3. Seroprevalence of MNV in mouse colonies

Original source	No. of positive sera / No. of sera			
	Genetic character			Total (facility)
	GMM[a)]	BC[b)]	CM[c)]	
CS[d)]	24/97	13/168	19/278	56/543 (10.3%)
NCS[e)]	43/135	2/7	0/8	45/150 (30.0%)
Unknown[f)]	9/49	2/3	0/0	11/52 (21.2%)
Total (Genetic character)	76/281 (27.0%)	17/178 (9.6%)	19/286 (6.6%)	112/745 (15.0%)

a) GMM: genetically modified mice. b) BC: in-house breeding colony of common inbred/outbred mice. c) CM: common inbred/outbred mice purchased from vendors. d) CS: commercial source. e) NCS: noncommercial source. f) Unknown: Original sources are unknown.

equivocal (*i.e.*, NS=2), they were interpreted as negative.

To design a pan-MNV specific primer, nucleotide sequence alignment was conducted with previously reported MNV subtypes located in the MNV conserved sequence region (Table 2). A new pan-MNV specific primer (primer # 2) was designed to amplify of the MNV protein VP1 gene using CLC DNA Workbench version 3.6.2 (CLC bio, Aarhus, Denmark) and was produced by Bioneer (Daejeon, Korea).

Viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, U.S.A.) from fresh feces of ten GMM (B6.CD1d$^{-/-}$) and 5 inbred (C57BL/6) mice maintained in facility B. Those B6.CD1d$^{-/-}$ colonies were known to be seropositive through regular microbiological monitoring. The C57BL/6 mice were wild-type mice purchased from vendors, and their feces were used as negative controls. Synthesis of cDNA was carried out using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Shiga, Japan). Reverse transcription reactions were conducted as follows: samples were incubated at 42°C for 60 min and predenatured at 70°C for 15 min. PCR was conducted using a PTC-100 Thermal Cycler (Bio-Rad, Hercules, CA, U.S.A.) with the following conditions: 40 cycles of 94°C for 30 sec, 56 or 60°C for 30 sec, and 72°C for 40 sec. The specific annealing temperatures were 56 and 60°C for the newly designed and previously reported primers, respectively. The product was separated via electrophoresis using a 2.5% agarose gel for 30 min at 100 V. After staining with 0.5 μg/m*l* ethidium bromide, amplified fragments were visualized with ultraviolet light using a gel documentation system (Gel Doc, Bio-Rad, Milan, Italy).

To further prove the existence of MNV, direct sequencing and a BLAST search were conducted. Among the amplified fragments, two products were selected using primer #2 (the newly designed primer in this study). Amplified MNV gene products were purified using a gel extraction kit (Qiagen), and nucleotide sequencing was conducted. The sequencing reaction was performed in an MJ Research PTC-225 Peltier Thermal Cycler using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit with AmpliTaq® DNA Polymerase (FS enzyme; Applied Biosystems). Amplified inserts were sequenced in both directions using forward and reverse primers for each amplified fragment. The sequences were compared to a DNA database using NCBI BLAST.

Of the 745 serum samples tested by ELISA, 112 (15%) were positive for MNV antibody, indicating widespread exposure to MNV in Korean research mouse colonies. The maximum OD value of positive sera was 3.892, with a minimum value of 0.105 and mean value of 1.039. The minimum value of total sera was 0.000, with a mean value of 0.191. Of the samples collected, 281 of the 745 (37.7%) samples were obtained from GMM. From these, 76 (27.0%) were positive, representing 67.9% of the total positive sera. Only 17 (9.6%) samples of 178 (23.9% of the total sample)

inbred/outbred strains bred in-house were positive. Finally, 19 (6.6%) samples from 286 inbred/outbred mice purchased from commercial vendors (38.4% of the total sample) were positive (Table 3). Of the samples obtained for this study, 543 samples came from mice purchased from commercial vendors, and 56 (10.3%) of these samples were positive. In contrast, 150 samples originating from mice from noncommercial sources (in-house breeding programs of inbred/outbred mice), and 45 samples (30.0%) were positive. Positive samples of animals originating from noncommercial sources were originally imported from a number of universities in the U.S.A. (Table 3). In both the F and N facilities, samples from all mice had antibodies against MNV. Both of these facilities are managed in a conventional manner. Because the number of mice kept under certain conditions was too small, we could not conclude that there is any tendency according to age, sex or strain of mice among the collected samples (data not shown).

RT-PCR was conducted to provide further evidence of MNV infection. Viral RNA was detected from fresh feces of ten B6.CD1d$^{-/-}$ mice when using three different types of primers, but it was not detected in fresh feces from five C57BL/6 mice. Fig 1 shows representative results from two samples. Partial sequencing of MNV obtained from a B6.CD1d$^{-/-}$ mouse demonstrated 100% sequence homology regardless of the primers used. Sequences were compared against known MNV sequences using a BLAST search. When using the newly designed primer (primer #2), there was very high homology of the cDNA sequences of the partially isolated MNV genes with murine noroviruses GV/CR7/2005/USA (accession no. EU004677; 99%) and GV/WU23/2005/USA (accession no. EU004668; 98%), specifically MNV-4 (accession no. FJ446719; 100%; data not shown).

In Korea, MNV has not been a major pathogen in laboratory mice; therefore, the virus was not routinely monitored prior to 2007. Until recently, MNV has been detected by RT-PCR and multiplexed fluorescent immunoassay (MFI). The MNV-1 MFI was almost 100% specific and sensitive in detecting anti-MNV-1 antibody in sera from experimentally infected mice [5]. Due to the lack of routine availability of MFI in Korea and the high associated cost, this study was conducted using commercially-available ELISA kits for MNV. Serological prevalence of 42,000 samples was previously reported by Henderson using ELISA [3]. Serological tests can produce false negative results due to null B cell immunity, failure of seroconversion or an immature immune system in young mice. To eliminate this possibility, mice with minimal to no seroconversion such as BALB/c-nu and SCID were eliminated from the total samples evaluated in this study. In the youngest seropositive mice in this study (5 weeks of age), we could not determine if seropositivity resulted from true MNV infection or passive transfer of maternal MNV immunoglobulin. RT-PCR for MNV may be an alternative diagnosis method for these animals, and a subset of positive samples was further confirmed for viral presence using RT-PCR.

According to the present study, there are MNV antibodies in mice housed in barrier-maintained facilities A to E (data not shown). These facilities have been in operation for 5 to 15 years. According to a previous study, a previously unknown MNV was found in 2003 [6]. Even a major laboratory facility (the Jackson Laboratory) started testing for MNV in 2006, and our monitoring center has been checking for this pathogen since 2007. Consequently, MNV-infected mice could have been carried into barrier facilities prior to this time.

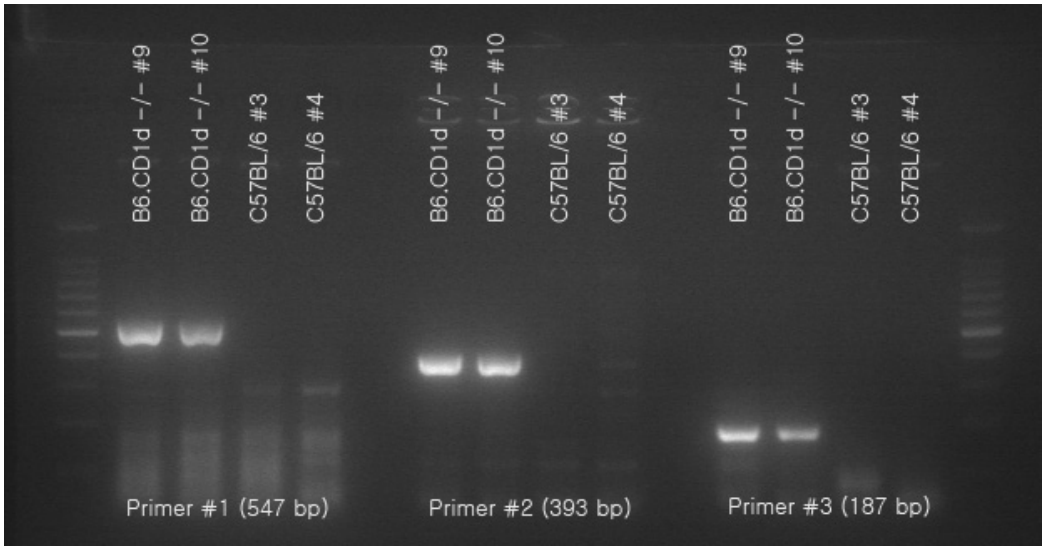

Fig. 1. Results of RT-PCR to identify MNV using various primers. Identical results were obtained when using three different types of primers. MNV was detected in B6.CD1d$^{-/-}$ mice (# 9 and # 10), but was not detected in C57BL/6 mice (# 3 and # 4).

Additionally, the rate of MNV infection was greater among the mice housed in barrier facilities than among mice in conventional environments (data not shown). We hypothesize that the rate of MNV infection depends on whether MNV-infected mice are bred or not. Highly MNV-positive facilities (B, F and N) were producing many infected mice. However, others did not. Therefore, we conclude that breeding is not major factor for high rates of MNV infection, whether the facilities are barrier or conventional.

Interestingly, samples from GMM had a 27% positivity rate for the MNV ELISA. Most of these animals originated from facilities in the USA. GMM were more often positive because regardless of their genetic alteration, they may not be as fully immunocompetent as non-manipulated inbred mice. Furthermore, many GMM may not be routinely produced and housed under barrier conditions. Common inbred/outbred mice purchased from SPF-grade vendors (facilities K and L) were not positive for MNV.

It is uncertain whether the serological test for MNV infection can detect seroconversion according to pan-MNV infection. Further study is needed to determine whether ELISA can detect seroconversion by pan-MNV infection through experimental super-infection of various MNV subtypes using RT-PCR to characterize each MNV subtype.

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